CELLULAR REQUIREMENTS FOR THE INDUCTION OF CYTOTOXIC T CELL RESPONSES *IN VITRO*

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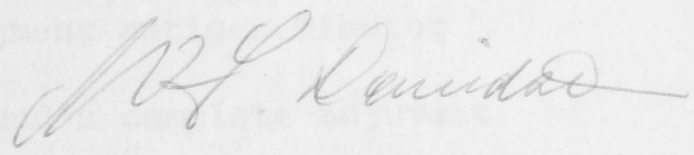
S T A T E M E N T

The experiments described in this thesis constitute my own original work and were carried out by myself, except in the individual instances stated.



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A B B R E V I A T I O N S

AT x BM	Adult thymectomised bone marrow reconstituted
B cell	Bone marrow-derived lymphocyte
BM	Bone marrow
C'	Complement
CML	Cell mediated lympholysis
CR	Complement receptors
CTL	Cytotoxic T lymphocytes
C.U.	Cytotoxic unit
DNP-BSA	Dinitrophenylated bovine serum albumin
DNP-MON	Dinitrophenylated monomeric flagellin
DTH	Delayed type hypersensitivity
Fab	Fragment antigen binding
FCA	Freund's complete adjuvant
FcR	Fc receptor
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
GC	Guinea pig complement
GVH	Graft versus host
H-2 complex	Mouse magor histocompatibility complex
HI	Heat inactivated
HVG	Host versus graft
Ia	I-region associated antigen
IFP	Intra-footpad
Ig	Immunoglobulin
IP	Intraperitoneal

Lad	Lymphocyte activating determinant
LD	Lymphocyte defined
LN	Lymph node
ln	Napierian logarithm ($\log_e$)
log	Common logarithm ($\log_{10}$)
2-ME	2-mercaptoethanol
MEM	Minimal essential medium
MHC	Magor histocompatibility complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocytes
PC	Peritoneal cells
PFC	Plaque-forming cells
RC	Rabbit complement
SD	Serologically defined
S.D.	Standard deviation
SRBC	Sheep red blood cells
T cell	Thymus-derived lymphocyte
TDL	Thoracic duct lymphocytes
θ	theta
UV	Ultra-violet

ANTILOGARITHMS (2-place)

$\log_{10}$	antilog
.00	1000
.05	1122
.10	1259
.15	1413
.20	1585
.25	1778
.30	1995
.35	2239
.40	2512
.45	2818
.50	3162
.55	3548
.60	3981
.65	4467
.70	5012
.75	5623
.80	6310
.85	7079
.90	7943
.95	8913
.99	9772

A B S T R A C T

At least three populations of cells are thought to be required for the induction of cytotoxic T cell responses to alloantigens *in vitro*. These include: a population of Ly $2,3^{+}$ cytotoxic T cell precursors, a lymphoid stimulator population and an adherent accessory cell population. There is some suggestion that a fourth population of Ly-1^{+} helper T cells may also be a necessary inclusion. Although the T cells responding to alloantigens have been well characterised there is still considerable debate concerning the identity of the cells which stimulate responses. Thus, while it is generally agreed that lymphoid cells are a better source of stimulator cells than non-lymphoid cells the relative capacity of B cells, T cells and macrophages to stimulate cytotoxic T cell responses remains contentious. Similarly, the identity and the function of the adherent accessory cell population is unresolved.

In this thesis an attempt is made to determine the relative stimulator activity of various subpopulations of lymphoid cells and to characterise the adherent accessory cell population. These comparative studies were facilitated by the use of a standardised MLC procedure and the expression of cytotoxic activity in terms of a derived unit - the cytotoxic unit.

Ontogenic studies and studies with adult lymphoid populations provided evidence that stimulator activity was a function of both mature and immature lymphoid cells and was not dependent on the presence of surface Ig, Ia or theta antigen, or Fc or complement receptors. In contrast to lymphocytes mature macrophages were poor stimulators. P815-X2 mastocytoma cells, L-929 cells and IgA plasmacytoma cells all

induced responses of magnitude similar to those observed with lymphocytes. Significant cytotoxic T cell responses were obtained in the absence of an I region difference between the stimulator and the responder population, suggesting that helper T cells which recognise Ia antigens are not an absolute requirement for the induction of cytotoxic T cell responses *in vitro*.

When mature, non-adherent, resting lymphocytes were used as a source of stimulator cells in primary MLC there was an additional and absolute requirement for an adherent accessory cell population. This cell population was present predominantly in spleen, bone marrow and foetal liver, and to a lesser extent in lymph nodes and the peritoneal cavity, but was absent from the thymus. The accessory cells were required for the induction rather than the maintenance of the response, functioned whether syngeneic or allogeneic with the responder population and were completely removed by treatment with carbonyl iron. Moreover, the observation that accessory cells could be replaced with L cells and factors produced by lymphoid cells cultured in the absence of antigen, suggested that this population featured in neither the presentation nor the processing of antigen and thus differed from the accessory cell population required for the induction of antibody responses *in vitro*.

The splenic accessory cell was further characterised as an $Ig^{+}\theta^{-}$ and tentatively $FcR^{\pm}$, $CR^{\pm}$, Ia^{-} cell which was not a mature macrophage. This data together with indirect evidence from ontogenic studies implied that the functional population was comprised predominantly of immature B cells, although the possibility that some accessory cells were immature FcR^{+} macrophages was not completely eliminated.

Although accessory cells were required for the induction of primary responses when resting lymphocytes were the source of stimulators this requirement was less critical when neoplastic stimulator cells or memory responder populations were used.

A model suggesting possible mechanisms by which cytotoxic T cell precursors, lymphoid stimulator cells and adherent accessory cells may interact in the induction of cytotoxic T cell responses *in vitro* was proposed. Briefly, this involved the recognition, by responder T cells, of alloantigens presented on the surface of "stimulator cells". It was suggested that only cells which were activated, and as a result of such activation, were able to produce inductive stimuli were able to function as "stimulator cells". The conversion of resting lymphocytes to "stimulator cells" was thought to be a function of the adherent accessory cell population.

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Preamble

In the past, the biology of the T lymphocytes has lagged well behind that of the B lymphocytes. The last five years, however, have seen the recognition of the functional heterogeneity of the T cell population, the complex interaction of T cells with each other and with B cells and macrophages, and perhaps most importantly, the central role of the products of the major histocompatibility complex (MHC) in T cell function. Today, this field is still highly speculative and there is little information regarding the nature of the antigens which stimulate the various subpopulations of T cells, the cellular requirements for stimulation and the types of receptors T cells use for recognition of antigen.

One subclass of T cells - the cytotoxic T cells - which for some years has been thought to play a prominent role in allograft rejection and immunity to some virus infections and possibly tumours, has proven a useful model for the study of the cellular and antigenic requirements for T cell induction. The following review of cytotoxic T cells is not intended to be all-inclusive. Rather, the aim is to present an overview with emphasis placed on some of the currently important issues and problems in the field. By way of introduction, and, more importantly, to emphasise the complexity of T cells as a class, it was considered worthwhile to briefly review the literature which suggests that T cells can be divided into functionally unique subsets, one of these being cytotoxic T cells.

1.1 Functional heterogeneity of the T cell pool

Historically, it was recognised that the immune response to foreign substances consisted of two basic components: (1) humoral immunity in which antibodies, characterised as immunoglobulin molecules, were synthesised and released into the circulation and (2) cell-mediated immunity which occurred independently of humoral immunity and was responsible for the delayed type hypersensitivity reactions, transplantation immunity and cellular resistance to certain viruses and bacteria. Subsequently, the effector population in humoral immunity was found to be the bone marrow-derived B lymphocyte (for review, see Miller and Mitchell, 1969) and in cell-mediated immunity the thymus-derived T lymphocyte (for review, see Miller and Osoba, 1967).

More recently, research has extended to the cellular requirements for the induction and control of these responses and it has become increasingly evident that T cells play a prominent role in this respect. Thus, T cells represent a very heterogeneous class of lymphoid cells whose diverse activities can be divided into the following categories:

(1) Helper/initiator

(2) Suppressor/regulator

(3) Inflammatory (GVH, DTH)

(4) Cytolytic.

Realisation of this functional heterogeneity has led researchers in the field to pose two fundamental questions: firstly, whether individual T cell functions are mediated by unique and separable subsets of T cells and secondly, if such differentiation does exist, whether it occurs before or after antigenic stimulation?

In the past, attempts to answer these questions have been hampered by the absence of both single cell techniques analogous to those used in the study of B cells and unique markers for individual T cells and their products. As a result of these limitations T cell subpopulations were

characterised indirectly and defined by criteria such as quantity of cell surface theta (θ) antigen (Shortman *et al.*, 1972a, Shortman *et al.*, 1975); size, density and electrophoretic mobility (for review, see Shortman *et al.*, 1975); homing characteristics and tissue distribution (Cantor and Asofsky, 1970; Cantor and Asofsky, 1972; Wagner, 1973; Tigelaar and Gorczynski, 1974); and sensitivity to adult thymectomy and anti-thymocyte serum (Kappler *et al.*, 1974; Cantor *et al.*, 1975). Taken collectively, data from these studies only suggested that following antigenic stimulation, individual functions may be performed by T cells that have different biological and physical properties.

More recently, however, with the use of T cell specific anti-Ly alloantisera (Boyse *et al.*, 1968; Itakura *et al.*, 1972; Shen *et al.*, 1975) and antisera to the Ir region associated (Ia) antigens (Murphy *et al.*, 1976) more direct evidence for the existence of functionally unique subsets of T cells has become available. Table 1.1 summarises data on the separation of T cells into various subclasses on the basis of these markers.

Thus, at least three of the four categories of T cell functions appear to be mediated by individual and separable subpopulations of T cells, namely Ly-1$^+$ helper cells (T_H), Ly2,3$^+$Ia$^+$ suppressor cells (T_S) and Ly2,3$^+$Ia$^-$ cytotoxic cells (T_C). T cells involved in delayed type hypersensitivity (DTH), although separable from cytotoxic and suppressor T cells cannot currently be separated from helper cells. The differences between the two subpopulations of cytotoxic T cells will be discussed in subsequent sections.

Further studies with Ly alloantisera have also indicated that helper (Cantor and Boyse, 1975a; Jandinski *et al.*, 1976), suppressor (Jandinski *et al.*, 1976) and cytotoxic (Cantor and Boyse, 1975a) T cells are functionally committed prior to stimulation with antigen.

TABLE 1.1

DIFFERENTIATION OF T CELLS ON THE BASIS OF CELL SURFACE MARKERS

T cell subset	Ly expression			Surface Ia	References
	Ly-1	Ly-2	Ly-3		
Helper/initiator (T_H)	+	-	-	-	Cantor and Boyse, 1975a Cantor *et al.*, 1975
Suppressor/regulator (T_S)	-	+	+	+	Herzenberg *et al.*, 1976 Jandinski *et al.*, 1976
Cytotoxic (T_C) allogeneic	-	+	+	-	Cantor and Boyse, 1975b
Cytotoxic (T_C) TNP modified self	+	+	+	-	Cantor and Boyse, 1976
DTH (T_D)	+	-	-	-	Huber *et al.*, 1976

In summary, it now appears that helper, suppressor and cytotoxic T cell functions are mediated by separate subpopulations of T lymphocytes which are committed to these functions prior to contact with antigen.

1.2 The history of cytotoxic T cells and their importance *in vivo*

1.2.1 Historical review

Much of the early work on tissue transplantation was carried out using malignant tumours. From such studies it was established that lymphocyte infiltration was associated with the pathology of graft rejection (De Fano, 1910). However, Murphy (1913, 1914) and Murphy and Taylor (1918) were the first to suggest that rejection of transplanted neoplastic tissue may be the result of an active immune response by the recipient.

Nearly twenty years elapsed before Gorer (1937, 1938), in a classic series of papers, defined histocompatibility antigens and thus established the genetic and immunological basis of rejection. At the same time, Medawar (1944, 1945) described the rejection of skin homografts in man and rabbits and formulated the general rule that transplantation immunity was the outcome of a systemic and not a local reaction. Medawar (1944, 1945) further proposed that the immunological mechanisms involved in graft rejection were specific and differed from those of conventional antigen-antibody reactions.

Support for this suggestion came later from histological studies performed by Kidd and Toolan (1950) which showed that infiltrating lymphocytes had a cytotoxic effect on tumours undergoing rejection. Similar findings were later reported by Waksman (1960) for allograft rejection.

If the small lymphocyte does in fact initiate the response to allografts, the injection of such cells would be expected to transfer this capacity to non-immune animals and lead either to sensitisation of the new recipient or to the breakdown of well established grafts in tolerant hosts. These expectations have been borne out, firstly, by experiments

involving adoptive transfer of tumour immunity with sensitised lymph node cells by Mitchison (1954; 1955) and secondly, by abrogation of neonatal tolerance to transplantation antigens by transfer of lymph node cells of histoidentical donors (Billingham *et al.*, 1956). The active population in such transfer studies was later identified as the small lymphocyte (Gowans, Gesner and McGregor, 1961; Gowans and Knight, 1964; Gowans and McGregor, 1965).

By 1961 there was therefore considerable evidence to suggest that specifically sensitised cytotoxic lymphocytes play a prominent role in both tumour and allograft rejection.

1.2.2 *In vitro* demonstration of cytotoxic T cells

The next major progression in the study of immunity to allografts and tumours was the development of *in vitro* methods for the assessment of the direct cytotoxic activity of immune lymphoid cells for donor target cells. Prior to 1960, evidence for cytopathic effects of lymphoid cells in graft rejection had been gathered, almost exclusively, from *in vivo* experiments where it was difficult to analyse the immune reaction at the cellular level.

The pioneers in the development of *in vitro* methods in this field were Govaerts (1960) and Rosenau and Moon (1961, 1962). The former author demonstrated that donor kidney target cells were specifically recognised and destroyed by TDL's from dogs bearing kidney allografts. Rosenau and Moon obtained similar results with BALB/c spleen cells from mice sensitised with L cells, as effectors, and L cells as targets. In contrast to Govaerts, these authors also demonstrated that target cell destruction by the effector population was independent of antibody or complement.

Similar studies soon followed in many laboratories (for review, see Perlmann and Holm, 1969). The methods employed for the detection of effector cells ranged from estimations of cell death (Govaerts, 1960;

Rosenau and Moon, 1962; Wilson, 1963) and cloning assays (Brunner *et al.*, 1966) to an isotope (^{51}Cr) release assay initially described by Holm and Perlmann (1967), and later adapted by Brunner and colleagues (1968), which has subsequently been universally adopted as the method of choice.

The lymphoid cells responsible for direct cytotoxic effects *in vitro* in the ^{51}Cr release assay have been shown to be T cells. Initially this was demonstrated indirectly using *in vivo* sensitised thymus cells (Cerottini *et al.*, 1970a) and then subsequently directly, using anti-θ serum and complement treatment of sensitised cells (Cerottini *et al.*, 1970b). The activity of cytotoxic T cells, as well as being specific was also shown to be independent of antibody and complement, normal T or B cells, antibody producing cells and macrophages (Cerottini *et al.*, 1970b; Cerottini *et al.*, 1971 ; Cerottini *et al.*, 1972).

These results, which were confirmed in several other studies (Blomgren *et al.*, 1970; Miller *et al.*, 1971; Sprent and Miller, 1972), clearly indicated that sensitised T cells capable of specifically and independently lysing cells syngeneic with those used for sensitisation, could be obtained from animals undergoing allograft rejection or GVH reactions.

Much less information is available in syngeneic tumour systems. The two systems most extensively studied are those involving murine leukaemia and sarcoma virus induced tumours in rodents (for reviews, see Shellam *et al.*, 1976; Tevethia *et al.*, 1976). Using these models several workers have demonstrated the presence of specific cytotoxic T cells in animals undergoing tumour rejection (Leclerc *et al.*, 1973; Djeu *et al.*, 1974; Shellam, 1974).

It should be emphasised at this point that cells other than cytotoxic T cells have since been found to mediate *in vitro* cytotoxicity in both allograft and tumour systems. Two main cell types are involved, namely, K cells and macrophages. K cells are lymphoid-like cells which lyse antibody-coated target cells in the absence of complement (for

reviews, see Perlmann and Holm, 1969, Cerottini and Brunner, 1974). Macrophages also have the capacity to lyse some antibody-coated target cells directly (Zighelboim *et al.*, 1973). Additionally they can be specifically armed by T cells (for reviews, see Lohmann-Matthes, 1976, Evans and Alexander, 1976) or activated (for review, see Nelson, 1969; Keller, 1976) to lyse target cells.

Thus, it is clear that cell-mediated cytotoxicity *in vitro* is by no means the expression of a single mechanism. It is pertinent, therefore, to establish the *in vivo* roles, if any, of the various cell populations which exhibit *in vitro* cytotoxicity.

1.2.3 The *in vivo* role of cytotoxic T cells

(a) Allograft and tumour immunity - The first evidence for the *in vivo* activity of immune T cells was provided by Sprent and Miller (1971, 1972), who showed accelerated and specific graft rejection in neonatally thymectomised (NTX) CBA mice following intravenous injection of a highly enriched population of CBA T cells immunised against the graft alloantigens. The T cells which were obtained from thoracic duct lymph were shown to contain cytotoxic T cells *in vitro*. The same population when mixed with P815 tumour cells bearing the relevant alloantigens prior to intraperitoneal injection into NTX mice was able to inhibit tumour growth. Essentially similar findings to those reported by Sprent and Miller have also been reported by Cerottini *et al.* (1972), and Freedman *et al.* (1972). These workers used heavily irradiated recipients rather than NTX recipients to demonstrate that alloimmune T cells alone protected CBA mice from P815 tumours and induced GVH reactions. It was also noted about this time that labelled immune T cells, when injected into animals bearing allografts, accumulated preferentially at specific graft sites (Lance and Cooper, 1972; Cerottini and Brunner, 1974).

Experiments of this kind strongly suggested that the cytotoxic T cells were the active component in rejection. It should be emphasised,

however, that the populations used for transfer of immunity, although enriched for cytotoxic T cells almost certainly contained other T cell subpopulations as well, for example, DTH cells which could cause activation of macrophages in the recipient host. The data certainly does not rule out a possible role for macrophages and K cells in the rejection process, although the existence of the latter at least in the Brunner and the Freedman systems is unlikely. Assuming that the predominant effector population is the transferred cytotoxic T cell, the question still remains whether these cells perform autonomously or in conjunction with host macrophages.

Adoptive transfer studies have also been performed in syngeneic tumour systems and several groups have shown that populations containing cytotoxic T cells can transfer specific immunity (Gorczynski, 1974; Shellam, 1974; Gorczynski and Knight, 1975a,b). As in studies with allografts and allogeneic tumours, a good correlation exists between the *in vitro* T killer activity of the transferred immune T cell population and its ability to cause tumour rejection *in vivo*. However, similar criticisms to those levelled at the allogeneic systems also apply to this one.

The data concerning the requirement for macrophages for the activity of cytotoxic T lymphocytes *in vivo* is conflicting. Studies by Giroud and others (1970) and Rouse and Wagner (1972) with allografts and Zarling and Tevethia (1973a,b), in a syngeneic tumour system, suggested that cytotoxic T cells were unable to cause allograft or tumour rejection in irradiated recipients in the absence of bone marrow (BM) cells. Their interpretation of these observations was that BM cells provided a source of macrophage precursors required for rejection. In contrast to these findings, reports from others (Nomoto *et al.*, 1970; Lubaroff, 1973; Freedman *et al.*, 1972) using essentially similar experimental systems have not shown the same requirement for BM cells for graft rejection by T cells.

One possible explanation for the discrepancy may be that sensitised T cells under optimal experimental conditions are autonomous as killers, whereas under less artificial conditions they may require co-operation with other effector cells for graft or tumour rejection to occur. There is, in fact, considerable evidence from *in vitro* studies that immune T cells can produce factors which specifically arm macrophages which in turn acquire cytostatic (Seeger and Owen, 1974) or cytotoxic activity for tumour cells (Alexander *et al.*, 1972; Lohmann-Matthes and Fischer, 1973). It is not known, however, whether the cells which produce arming factors are cytotoxic T cells or, more importantly, whether such a system operates *in vivo*, although there is some evidence that macrophages may be important in tumour rejection and control of metastases (Gorer, 1961; Evans, 1973; Eccles and Alexander, 1974) and possibly allograft rejection (Dyminski and Argyris, 1969).

To date, there is little direct evidence for an *in vivo* role of K cells in tumour or allograft rejection. A number of reports (Spong *et al.*, 1968; Clark *et al.*, 1968) have shown that transfer of immune serum causes first-set rejection in a kidney graft and that this is usually associated with an infiltration of mononuclear cells. Maclennan (1972) has since shown that antibody dependent killer cells can be found in the mononuclear infiltrate of a transplanted kidney that is undergoing chronic rejection. Such findings suggest, but do not confirm a possible role of K cells *in vivo*. A more recent report (Ramshaw, 1975) has demonstrated that the injection of a highly purified population of K cells together with antibody coated P815 tumour cells into the footpads of BALB/c mice resulted in the failure to produce measurable tumour growth, as compared to controls receiving untreated or antibody-coated P815 alone or K cells + untreated P815. However, comparable tumour growth was observed when either untreated or antibody coated P815 cells alone were injected into footpads. This less artificial approach indicates

that the presence of antibody coated cells does not result in a more rapid recruitment of effector cells. It should also be noted that the time course for P815 tumour regression in the footpad parallels that of the generation of cytotoxic T cells in the draining lymph node.

(b) Viral immunity - The ^{51}Cr isotope release assay as well as being used for the detection of cytotoxic cells in tumour and allograft systems, has also been exploited in the study of T cell responses to virus infections. The presence of cytotoxic cells in animals with active viral infections has been reported by many groups in the past (Speel *et al.*, 1968; Lundstedt, 1969; Oldstone and Dixon, 1970; Marker and Volkert, 1973). Recently, more detailed characterisation of the specific effector cells in a variety of systems employing lymphocytic choriomeningitis (LCM) virus (Cole *et al.*, 1973; Doherty *et al.*, 1974) and ectromelia virus (Gardner *et al.*, 1974a,b) have indicated that T cells acting alone kill virus infected targets *in vitro*.

Evidence from *in vivo* protection studies with transferred immune cells strongly suggests that cytotoxic T cells play a significant role in the immunity to both ectromelia (Kees and Blanden, 1976) and LCM viruses (Zinkernagel and Welsh, 1976). However, as with the tumour and graft systems, it is not known whether *in vivo*, cytotoxic T cells act alone or in concert with macrophages. There is no evidence that K cells play a part in immunity to either of these viruses.

K cells do, however, appear to have activity in some *in vitro* virus models. These include herpes simplex virus (Ramshaw, 1975; Rager-Zisman and Bloom, 1975), measles virus (Labowskie *et al.*, 1974), and rubella virus (Steele *et al.*, 1973). It still remains to be determined how important this form of immunity is in the recovery from viral infections.

1.2.4 General conclusions

(a) Cell mediated immune responses *in vivo* to allografts and some tumours and viruses can be shown to be mediated *in vitro* by three cell types, notably, cytotoxic T cells, macrophages and K cells. Each of these effector populations performs, *in vitro* at least, independently of the others.

(b) The functional role of these effector populations *in vivo* is highly controversial. Although the immunity conferred in allograft, tumour and virus systems following transfer of immune T cells containing cytotoxic T cells correlates well with the *in vitro* killer activity of the transferred population the extent to which cytotoxic T cells alone are responsible for immunity *in vivo* is still unclear.

It should be emphasised that many of the transfer studies performed - particularly those in allograft and tumour models - have employed either cytotoxic T cells from animals undergoing GVH reactions or premixtures of such cells with tumour or grafted cells. Injection of such purified and highly potent populations may result in an exaggeration of the actual activity of the cytotoxic T cells. In the normal situation of HVG reactions, such a potent population of cytotoxic T cells may not in fact exist or may never concentrate in such extreme numbers at the reaction site. Under less artificial conditions - *in vivo* - cytotoxic T cells may in fact require co-operation with other effectors, particularly macrophages, for graft and tumour rejection or viral immunity to occur. Pretreatment of the immune T cell population with relevant anti-Ly alloantisera prior to transfer to recipients may allow a more precise determination of the *in vivo* role of the T_C subset in the future.

Transfer studies with macrophages and K cells have generally been unsuccessful. This failure is probably a reflection of the inability of these effector populations to recirculate in such a differentiated form rather than an indication of their lack of involvement *in situ*.

To conclude: cytotoxic T cells, macrophages and K cells probably all function *in vivo*. The extent to which any one population is active may depend on parameters such as the type of antigen, the site of antigen localisation, the presence or absence of circulating antibody and the period during the immune response that is studied.

1.3 The *in vitro* generation of cytotoxic T cells

The preceding sections have considered the *in vivo* generation of cytotoxic T cells following challenge with allo-, tumour and viral antigens. Further evaluation of the cellular and genetic requirements for the induction of cytotoxic T cell responses has been facilitated by the use of *in vitro* culture techniques. Until very recently *in vitro* models of allograft rejection have received the greatest consideration and the following discussion will therefore concentrate almost entirely on these.

1.3.1 The mixed leukocyte reaction

By the end of 1963 it had become increasingly clear that a large number of antigens were capable of activating lymphocytes. Since it was also known that lymphocytes contain on their surfaces a number of important transplantation antigens, it seemed likely that mixtures of lymphocytes from unrelated individuals or unrelated species might stimulate each other. That blast cells do appear in mixtures of lymphocytes from two unrelated individuals was first clearly shown in humans by Bain *et al.* (1964) and Bach and Hirschhorn (1964) and then later in mice (Dutton 1965, 1966). The complex phenomena occurring when lymphocytes are cultured together later became known as the mixed leukocyte reaction (MLR) and the culture as the mixed leukocyte culture (MLC).

The major disadvantage of the original method was the difficulty it posed in assessing the individual contributions of the two cell populations to the reaction. For this reason, the two-way MLC has been largely replaced by its one-way modification where normal lymphocytes (responder population) are cultured with unrelated lymphocytes

(stimulator population) which have previously been irradiated or mitomycin C treated to abolish mitotic activity (Cepellini *et al.*, 1965; Bach and Voynow, 1966). The response in MLC is quantitated by measuring the incorporation of radioactive thymidine by the dividing responder population. The MLC has been used extensively for clinical matching of donor and recipient for transplantation and is generally considered to represent the initial or sensitising phase of the immune response to allografts *in vivo* (for references, see Bach, 1974; Bach *et al.*, 1975).

Further evidence for the immunological nature of the MLC was provided by the observation of Häyry and Defendi (1970) that immunologically specific cytotoxic cells were also generated in MLC. These cytotoxic cells were subsequently identified as T cells by the use of purified responder populations (Wagner, 1971; Häyry *et al.*, 1972; Feldman *et al.*, 1972) and abrogation of the response following treatment of the responder population with anti-θ serum and complement (Wagner *et al.*, 1972).

In general, the cytotoxic T cells generated *in vitro*, particularly in allograft systems, appear to be physically and functionally very similar to those generated *in vivo* (for review, see Cerottini and Brunner, 1974). The development of cytotoxic T cells *in vitro* would therefore appear to represent an *in vitro* analogue of the late, destructive phase of the immune reaction to allografts *in vivo*.

1.3.2 The H-2 complex

Following the foundation studies of Gorer and Snell, it was clear by the middle of the twentieth century that, in mice, a class of genes coded for alloantigens and that these alloantigens were responsible for rejection of incompatible neoplastic and normal tissue grafts (for references see Klein, 1975a). The antigens responsible for tissue compatibility were summarily designated by Snell (1948) as histocompatibility antigens and the genes coding for these structures as

THE H-2 COMPLEX

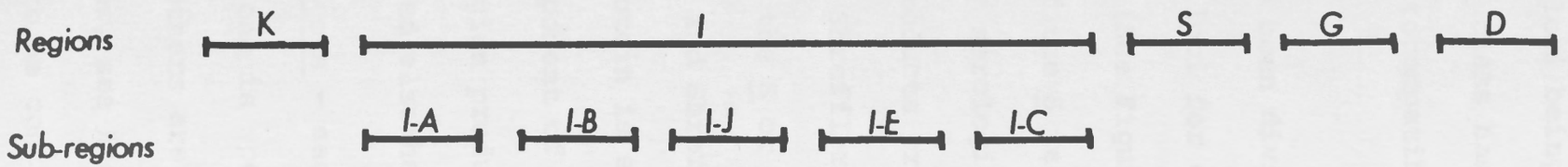

Figure 1.1 - Diagrammatic representation of the major histocompatibility complex in the mouse. The complex is divided into five regions with the I region further divided into five sub-regions (Klein, 1975a,b; Shreffler *et al.*, 1976; Murphy *et al.*, 1976).

histocompatibility (H) genes. Differential susceptibility of mice to grafts led Counce and co-workers (1956) to conclude that there were two types of H-genes: major (strong) and minor (weak). Originally, H-2 (coding for the first H-Ag discovered) was the sole representative of the strong category; all other H-loci being of the weak category. Today this still holds true, but the H-2 class has been found to be multigenic and is now known as the major histocompatibility complex (MHC) (or H-2 complex) of the mouse.

The MHC in the mouse has been divided into five regions: K, I, S, G, D, on the basis of marker loci for each region (Klein *et al.*, 1975a; Shreffler and David, 1975). (See Figure 1.1.) Products of each of these regions with the exception of the S region are expressed on the cell surface and are detectable by serological methods. The S region codes for two loci Ss and Slp whose products are secreted into the serum (Shreffler and Owen, 1963; Passmore and Shreffler, 1970). So far little is known about the products of either the S or the G regions, although recent studies by Meo *et al.* (1975) and Shreffler *et al.* (1976) have strongly implied that the mouse Ss protein is structurally and immulogically homologous to the fourth component of human complement (C4). Further properties of the S and G region products will not be discussed here but have been extensively reviewed elsewhere (Klein, 1975a,b; Shreffler, 1976).

(a) H-2K and H-2D antigens - each H-2K or H-2D region codes for a series of antigens of which one is specific for the given haplotype (private antigen), and the others are shared by two or more haplotypes (public antigens) (for review, see Klein, 1975b). Both private and public H-2 antigens appear to be controlled by the same genes and may be physically associated in the cell membrane, possibly on the same molecules (Hauptfeld and Klein, 1975).

(b) Ia antigens - Ia antigens are genetically controlled by the region between K and S, namely, the I region. Ia antigens are controlled by at least four or five loci in the I region, notably IA, IB, IJ, IC and perhaps IE (Shreffler and David, 1975; Sachs *et al.*, 1975; Murphy *et al.*, 1976). Individual Ia antigen subregions redistribute in the membrane independently of both K and D antigens and each other (Hauptfeld *et al.*, 1975; Klein, 1975b). Ia antigens, which can also be divided into private and public classes, have been demonstrated on a high proportion of B cells, some macrophages, epidermal cells and teratocarcinoma cells and a small proportion of T cells (for review see Hammerling, 1976).

Further physical and biochemical properties of the K, D and Ia antigens will not be discussed here, but have been reviewed extensively elsewhere (Klein, 1975a,b; Klein and Hauptfeld, 1976; Hess, 1976; Cullen, 1976).

1.4 Genetic control of MLR

Lymphocyte activation, reflected by blast transformation, can be effected by a number of cell surface antigens. These antigens which include major and minor H determinants and MLR determinants have been termed lymphocyte activating determinants (Lad's) by Festenstein and Démant (1973) and lymphocyte defined (LD) antigens by Bach *et al.* (1972a,b).

(a) Minor H antigens - The data from MLC combinations differing at single minor H loci are contradictory. Using combinations involving differences at H-1, H-7, H-8, H-Y and H-9 in two-way MLC, Dutton (1966) and Rychlikova and Ivanyi (1969) failed to observe stimulation, whereas Adler *et al.*(1970) and Mangi and Mardiney (1970), using one-way MLC observed low but significant stimulation in combinations differing at H-1, H-3 and H-4. Stimulation has also been observed for antigens controlled by the Thy-1 locus (Peck and Click, 1973). The discrepancies observed may be a result of the insensitivity of the MLC technique. Thus all minor H antigens may cause weak, but as yet undetectable

stimulation. To date, no systematic study on the role of these antigens in lymphocyte activation has been attempted.

(b) Mls locus determinants - Marked stimulation in MLC is observed with lymphocytes from mice differing at the Mls locus (mouse minor MLC stimulating locus). This locus was first described by Festenstein (1966) who observed that H-2^d identical BALB/c and DBA/2 mice reacted in MLC. The Mls locus thus segregates independently of H-2 and has at least four alleles: Mls^a, Mls^b, Mls^c, Mls^d (Festenstein, 1974). The Mls determinants have not been detected serologically but it is known that they are on B cells and probably macrophages, but not T cells (von Boehmer and Sprent, 1974; Schirrmacher *et al.* 1975).

(c) Major H antigens - In contrast to the weak stimulation observed with minor H antigens, MLC antigens cause strong proliferative responses in MLR.

The tacit assumption of early workers (Dutton, 1965, 1966; Huemer *et al.*, 1968) was that classical H-2 antigens defined by serological methods were responsible for the reaction. Following the discovery in man, by Yunis and Amos (1971), that MLC responsiveness could be separated from the two HLA loci coding for serologically detectable antigens, Rychlikova *et al.* (1970, 1971) reported that MLC stimulation in the mouse was associated almost exclusively with the K end of the H-2 complex. Bach *et al.* (1972a,b) using recombinant mice with crossovers occurring between the K and the I regions (AQR and B10.A(4R)) found that the I region rather than the K region was responsible for the "K end" stimulation. These findings were later confirmed by Meo *et al.* (1973b) using A.TL, A.TH and B10.A(4R) strains of mice.

So far, three lymphocyte activating determinants (Lad's) have been reported in the region between K and D. The strongest of these is associated with the IA region (Meo *et al.*, 1973a). The strong stimulation observed by many investigators with strain combinations differing at the

entire central segment of the H-2 complex can probably be attributed to antigens coded in this region (Bach *et al.*, 1972a; Widmer *et al.*, 1973c; Meo *et al.*, 1973b; Fathman *et al.*, 1974; Lonai and McDevitt, 1974). A second, weaker Lad locus has also been mapped to the I region (Bach *et al.*, 1973b). The relationships of the Lad loci to the Ir-A, Ia and H-2I loci mapping in the same region is unclear. There is some evidence, however, that Ia antibody (no complement) can block the mixed lymphocyte reaction at the stimulator level, suggesting that Lad's and Ia determinants may be either identical or closely associated on the cell membrane (Meo *et al.*, 1976). The third Lad locus which also codes for a weakly stimulating antigen has been formally mapped between Ss and D (Widmer *et al.*, 1973b).

The involvement of the H-2K and H-2D regions in MLR is a controversial one. Although both K and D region differences can result in significant stimulation in some strain combinations (Bach *et al.*, 1972a,b; Meo *et al.*, 1973b; Plate, 1974; Nabholz *et al.*, 1974), it has been argued that this stimulation is a result of antigens coded by Lad loci residing in the K and D regions but distinct from H-2D or H-2K. Recent data from studies with H-2 mutants suggest that such an explanation is unlikely.

Investigations with four H-2 mutants - CBA H-2^{ka} (Blandova *et al.*, 1975), B6.C-H-2^{ba} (Bailey *et al.*, 1971), B6-H-2^{bd} (Egorov and Blandova, 1968) and B10.D2-H-2^{da} (see Klein, 1975b) suggest that a single point mutation can be responsible for simultaneous changes in MLR, CML and histocompatibility (Widmer *et al.*, 1973a; Klein *et al.*, 1975b; Nabholz *et al.*, 1975a; Melief *et al.*, 1975).

The H-2^{ba} mutant which does not differ serologically from its congenic partner, B6-H-2^{b}, is one of nine mutants occurring at the hypermutable Z1 locus which has been mapped to the K end of H-2 (Melief *et al.*, 1975). That these mutants involve a single genetic element in H-2K

has been established by the demonstration of classical codominant transmission and by the results of complementation tests (for references, see Melief *et al.*, 1975). Thus, a mutation at a single locus results in reciprocal stimulation in MLR, generation of cytotoxic T cells, graft versus host reaction and skin graft rejection between B6 -H-2^{b} and its congenic partner B6.C-H-2^{ba}. There are several possible explanations for these findings:

(1) The antigens coded for by the Z1 locus may stimulate both MLR and CML responses.

(2) The cytotoxicity and skin graft rejection observed may be the result of recognition of I region determinants rather than K region determinants by cytotoxic T cells. This explanation is unlikely as Nabholz *et al.* (1975a) used PHA-stimulated target cells which are known not to display I region associated antigens.

(3) A change in the H-2K locus may affect the expression of MLR determinants coded for in the I region, although there is no evidence to support an interaction of this type.

(4) Two mutations may have occurred in the B6.C-H-2^{ba} mouse - one in the H-2K region (Z1 locus) and a second in the I region.

Although the last alternative may be a possibility in the H-$2K^{b}$ mutants, it certainly does not explain the reactivity of the CBA H-2^{ka} mutant where the mutation has been convincingly mapped to the K rather than the I region of the H-2 complex (Klein *et al.*, 1975b).

Alternatives (1) and (3) therefore seem to be the most likely explanations of the mutant studies. Further genetic, and more importantly, biochemical, characterisation of the MHC products involved will hopefully help to distinguish between these alternatives.

In summary, loci coding for the strongest Lad's map to the IA subregion of the H-2 complex. Currently, there is no convincing evidence to refute the existence of loci in the H-2K and H-2D regions coding for further, weaker Lad's as well. One interpretation of the mutant data would suggest that the Lad's coded in the K end, at least, cause both MLR and CML activity.

1.5 Genetic control of CML

(a) The effector phase - A number of reports from different laboratories show that in the mouse the determinants detected by killer cells in CML are controlled by loci very closely linked or identical to the H-2K and H-2D regions controlling the classical, serologically defined MHC antigens (SD antigens) (Alter *et al.*, 1973; Abbasi *et al.*, 1973; Nabholz *et al.*, 1974).

Further evidence suggesting the identity between the H-2K and H-2D antigens and the target antigens came from studies employing hyperimmune anti-H-2 sera with defined specificity (Brunner *et al.*, 1968; Bonavida, 1974; Nabholz *et al.*, 1974). Such sera were found to block CML only when they were directed against the H-2K or H-2D incompatibility against which the cytotoxic cell was active.

In apparent contradiction to the blocking data Berke *et al.* (1972a) observed cytotoxicity in the absence of serologically defined differences. Using a different experimental approach, early studies by Brondz and Goldberg (1970) showed that while target cells from the graft donor strain were destroyed *in vitro* by recipient spleen cells, target cells from an unrelated haplotype sharing several public H-2 specificities with the sensitising strain were not affected. In contrast, antiserum raised against either target was cross-reactive. Similar observations have subsequently been made by other groups (Forman and Möller, 1974; Lake *et al.*, 1974). In more recent studies Brondz's group has further demonstrated, using both *in vivo* and *in vitro* models, that cytotoxic T cells do not

recognise public H-2 specificities but react selectively either to private H-2 specificities or to serologically silent products of H-2K or H-2D closely linked to private specificities (Brondz *et al.*, 1975).

Similar observations have also been made in other systems, for example, cytotoxic T cells sensitised against virus infected syngeneic cells will only lyse virally infected targets sharing the same private H-2 antigen as the donor (Gardner *et al.* 1975). The same holds true for other syngeneic systems, namely TNP (Shearer, 1974), minor histocompatibility antigens (Began, 1974) and the H-Y system (Gordon *et al.*, 1975).

There is also evidence which suggests that the antigenic determinant recognised by cytotoxic T cells is serologically silent. This derives mainly from studies with the H-$2K^b$ mutants, where some mutations have been found to change CML determinants qualitatively while they leave the serologically detectable determinants, both private and public, intact (Bailey *et al.*, 1971; Berke and Amos, 1973a; McKenzie *et al.*, 1976). However, this feature seems to be unique to the H-2^b haplotype since SD differences have been observed between original strains possessing other haplotypes (H-2^d and H-2^f) and their respective mutants (Dishkant *et al.*, 1973; Egorov, 1974).

The failure to recognise SD differences in the H-2^b mutants was probably not the result of lack of generation of helper T cells through possible Ir (immune response) gene effects, as some immunisation procedures were performed on F_1 hybrid mice where another genetic background was introduced (McKenzie *et al*, 1976). It is notable, however, that the antibody response to the private H-2D antigen, H-2.2, appears to be under multiple gene control (Lilly *et al.*, 1973) so that assuming the private antigens are equivalent to the CML antigens, the lesion need not map to the Ir region. If the inability to make antibody to the H-$2K^b$ mutants is a genetic one it is not confined to B6 mice as (C3H x DBA/2)F1 mice were also unable to detect differences between the mutants and their congenic

partner (McKenzie *et al.*, 1976). Attempts with rats have also proven unsuccessful (for references see Nabholz *et al.*, 1974).

Studies with humans also indicate that cytotoxic T cells do not recognise serologically determined antigens. Thus, Bach *et al.* (1976) have found that human cytotoxic T cells sensitised to lyse cells from the stimulating cell donor can also specifically lyse target cells from a third party not sharing any serologically detectable cross reactivities with the stimulating cell donor.

In summary, the relationship between the antigens recognised by cytotoxic T cells and the antigens defined serologically is not clear. It seems to be generally accepted that public H-2 antigens are not recognised or are recognised only weakly by cytotoxic T cells. Whether the target antigen is, in fact, a serologically defined private H-2K or H-2D moiety or a serologically silent H-2K or H-2D moiety is as yet unresolved. Further studies with the $\underline{\text{H-2}}^{da}$ mutant may help to clarify this point.

As mentioned in the previous section, I region differences alone induce strong proliferative responses in MLR. In early studies several groups reported that despite such strong proliferative responses only weak CML activity could be detected (Alter *et al.*, 1973; Meo *et al.*, 1973a,b; Schendel and Bach, 1974; Nabholz *et al.*, 1974). This observation was the more difficult to understand because an I region difference between donor and recipient had been shown to be sufficient for the rapid rejection of skin grafts (Klein *et al.*, 1974a; Hauptfeld *et al.*, 1974). The question therefore arose whether the failure to detect cytotoxic T cells directed against I region determinants was due to the fact that killers directed against these determinants were genuinely absent or whether the targets used in the previous studies - mainly PHA induced blasts and macrophages - did not express the relevant antigens.

Klein *et al.* (1974b) were the first to show that CML across the I region could be obtained if killer cells were produced *in vivo* by skin grafting and LPS blasts were used as targets. More recently, Wagner *et al.* (1975a) and Nabholz *et al.* (1975b) have confirmed these results with MLC activated cytotoxic T cells. Using A.TL/A.TH strain combinations both groups observed significant, but weak, cytotoxicity against LPS but not PHA induced blasts as compared with T_C raised against a complete H-2 difference. It is not known whether these quantitative differences in cytotoxicity to K, D and I region determinants reflect a weak response to I region determinants or an inadequate target which only expresses some of the I region controlled CML determinants.

Wagner's group mapped the loci controlling the stimulating determinants to the IA subregion, whereas the data from Nabholz's group suggested at least two genes between the K and the D regions and possibly an extra - H-2 locus control the determinants detectable by the cytotoxic T cell population. Although Nabholz *et al.* showed that 60-80% of LPS blasts were lysed with anti-Ia serum and complement whereas this treatment had little effect on PHA blasts, the relationship between the I region CML determinants, the MLR stimulating determinants and the serologically defined Ia antigens is still unknown. The solution will probably only be found when characterisation of the relevant molecules complements functional assays.

To summarise: the target antigens recognised by cytotoxic T cells appear to be predominantly controlled by the H-2K and H-2D regions of the H-2 complex. Weak, but significant responses to I region coded determinants have also been reported.

It is notable that cytotoxic T cells are also able to recognise viral, tumour, xenogeneic, and minor histocompatibility antigens. These responses will be discussed in detail in subsequent sections. So far no cytotoxic activity has been detected against Mls controlled antigens

(Röllinghoff *et al.*, 1975; Lilliehook *et al.*, 1976).

(b) The induction phase - Data reviewed in the two previous sections considered collectively indicates non-identity between the antigens which cause maximal proliferation (I region coded) in MLR and those which are optimally recognised as target antigens (H-2K or H-2D coded) by cytotoxic T cells. This dichotomy has raised two fundamental questions: (1) Is the recognition of both antigens a requirement for the induction of cytotoxic T cells and, (2) if so, do separate subsets of T cells recognise these antigens? The past three years have seen many attempts to answer these questions.

Pioneer mapping studies with recombinant mice by Alter *et al.* (1973) suggested that in some MLC combinations, notably those involving the recombinant mouse strains AQR and B10.A which share the I region of the H-2 complex, but differ at the K region, no significant proliferation or generation of cytotoxicity was observed. Further experiments by the same group demonstrated that both proliferative and cytotoxic responses could be induced by the addition of a second stimulator population, B10.T(6R) which differs from the responder population only in the I region (Schendel *et al.*, 1973). Complementary results were obtained in similar studies with human lymphocyte combinations (Bach *et al.*, 1973b; Eijsvoogel *et al.*, 1973). The conclusions drawn from these studies were firstly, that recognition of both LD (I region coded) and SD (K and D region coded) differences was a prerequisite for the generation of cytotoxic T cells and secondly, that these antigens were probably recognised by different T cell subpopulations.

It should be emphasised, however, that the lack of MLR and CML responses to H-2K or H-2D coded antigens in the absence of I region differences is not a univeral one. For example, B10.G mice respond well to B10.T(6R) mice which differ only at H-2D (Schendel *et al.*, 1973). Similarly, A and A.TL strain combinations differing at K and Ss and A.TH

and A.SW combinations differing only at the D region also cause significant and bi-directional generation of cytotoxic activity (Nabholz *et al.*, 1974). Also, as mentioned in previous sections, all of the K and D region mutants led to strong CML when co-cultured with their respective congenic partners (Klein *et al.*, 1975b; Forman and Klein, 1975; Melief *et al.*, 1975). Moreover, following improvements in their culture techniques, more recent reports from Bach's group have shown that significant CML is generated with the original B10.A/AQR combination (Schendel and Bach, 1974). The further addition of B10.T(6R) cells, however, does significantly enhance the response. Similar results have also been obtained in *in vivo* studies (Sollinger and Bach, 1976) where AQR mice were shown to be fully capable of rejecting B10.A thyroid grafts, although rejection was found to occur more rapidly when B10.T(6R) lymph node cells were injected at the time of grafting.

In order to save the original SD-LD concept, it was then argued that the present delineation of K and I regions is arbitrary so that weak LD type loci may exist in the K region as presently defined by the existing recombinants. In an attempt to prove this point Schendel and Bach (1974, 1975) heat-treated or UV-treated the B10.A stimulator population. These techniques have been reported to inactivate LD antigens (Eijsvoogel *et al.*, 1973) whilst leaving SD antigens intact (Lindahl-Keissling and Safwenberg, 1971; Lafferty *et al.*, 1974a). The outcome of these experiments was that either heat- or UV-treatment of the B10.A population completely abrogated the CML response while the combination of treated B10.A populations and mitomycin C treated B10.T(6R) cells restored responses to their original levels. It is also notable that one of these reports (Schendel and Bach, 1975) also contained evidence that Mls differences are as effective as I region differences in enhancing responses.

Collectively, these results were taken by the authors as proof that LD differences, which can be coded in either the H-2K or the H-2I regions of the H-2 complex or alternatively the Mls locus are an absolute requirement for the induction of cytotoxic T cells. Alternate interpretations of the foregoing reports will be discussed in a later chapter.

In summary, the plethora of data which has accrued from studies with recombinant, congenic and mutant mouse strains, whilst conclusively demonstrating that recognition of strong MLR determinants (coding in the I region) is not a corequisite for induction of CML has nonetheless not eliminated the possibility of a requirement for recognition of cell surface antigens in addition to target antigens. Whether combined recognition of target and non-target antigens actually occurs and is an absolute requirement for induction of CML or alternately only serves to implement a helper mechanism is as yet unknown. Moreover, the final answer will probably not come from studies with genetic recombinants, as it may never be possible to absolutely separate genes coding for target antigens from those coding for other membrane antigens. However, the problem can be considered from a different aspect.

On the basis of a report by Cantor and Asofsky (1970, 1972) that functionally different subpopulations of T cells collaborate in GVH reactions, it was suggested initially by Bach *et al.* (1972a,b) and later by Wagner (1973) that MLR response and killer precursor cells belong to different subpopulations and that the collaboration between these, possibly triggered by different genetic incompatibilities, may be required for killer cell production.

Results from absorption studies (Bach *et al.*, 1973a) with allo-immune human lymphocytes are consistent with the existence of two separate populations of T cells, one responding to LD differences by proliferation and not adhering to a monolayer of the sensitising cells

and the other, the cytotoxic T lymphocytes which do adhere to the monolayer. Similar results were also obtained with unsensitised cells but neither experiment provided evidence for collaboration between the two subpopulations.

Further evidence that the cells proliferating in response to strong LD-type differences may be separable from cytotoxic cells comes from studies with T cell populations activated against K region determinants. It has previously been shown that the activity of killer cells with unknown specificity can be assayed by incubating them with an agglutinating lectin such as PHA or con A and a sensitive target (Stavy *et al.*, 1972; Asherson and Fergula, 1973; Forman and Möller, 1973). Using this technique Röllinghoff *et al.* (1975), and Dennert (1976), were able to show that T cells activated by Mls locus determinants are not cytotoxic.

Similarly, in humans strong support for the independence of the proliferating cells generated in the MLC reaction against LD differences and cytotoxic T cells comes from a recent report by Mawas *et al.*, 1975. In this study, patients suffering from primary immuno-deficiencies either lacked MLC responsiveness or had defects in killer cell induction or both, indicating that these two functions may be independent of one another.

Early evidence for collaboration between T cell subsets in the induction of cytotoxic T cells came from studies similar to those of Cantor and Asofsky. Thus the combination of thymus and limiting numbers of peripheral,T responder cells was found to produce MLR and CML responses far exceeding those of either population alone or the values expected from a simple additive effect. (Cohen and Howe, 1973; Wagner, 1973; Häyry and Anderson, 1974). The synoptic interpretation of these experiments was that peripheral T cells provide the major source of precursor cells of cytotoxic lymphocytes, while thymocytes perform mainly a helper or amplifier function.

More recently, the T cell populations responding in MLC have been separated on the basis of Ly markers into two subsets, namely, Ly-2,3$^+$ and Ly-1$^+$ cells (Cantor and Boyse, 1975a,b). As discussed in an earlier section, the precursor cytotoxic cells belong to the Ly-2,3$^+$ subset. The maturation of these cells was shown to be amplified by Ly-1$^+$ cells which did not themselves contribute to the killer cell pool. This amplification was abolished by excluding the Ia$^+$ cells from the stimulator population during MLC, suggesting that amplification was due to the selective recognition of I region determinants by Ly-1$^+$ cells (Cantor and Boyse, 1975b). It should be stressed, however, that significant amplification was only evident when Ly-2,3$^+$ cells were used in limiting numbers (cf. thymus/peripheral T cell experiments). Thus, optimal numbers of Ly-2,3$^+$ cells alone could generate substantial killer activity.

It is also noteworthy that in MLC's involving a complete H-2 difference removal of Ly-1$^+$ responder cells only caused a 40 per cent reduction in thymidine uptake, implying that Ly-2,3$^+$ cells do divide in MLC. On the other hand, Ly-1$^+$ cells account for almost all of the proliferative response in MLC's involving I region incompatibility only (Cantor and Boyse, 1975a).

To conclude, there is still considerable debate regarding the genetic requirements for induction of cytotoxic T cells. It would appear, though, that under optimal culture conditions, the recognition of H-2K or H-2D coded determinants by Ly-2,3$^+$ cells is sufficient for the generation of substantial cytotoxic activity. The response may be further amplified by Ly-1$^+$ helper cells responding principally to antigens coded in the I region. So far there is no evidence for the absolute requirement for T help that has been shown for the production of antibody to most antigens (for review, see Miller and Osoba, 1967).

1.6 Generation of cytotoxic T cells against other cell surface antigens

In addition to allogeneic cells, precursors of cytotoxic T cells are known to respond to two other categories of stimulator cell:

(a) Xenogeneic cells

(b) Syngeneic (or allogeneic) cells bearing membrane associated antigens in addition to MHC coded antigens.

(a) Despite early reports that MLR in xenogeneic combinations was either weak or non-existent (Wilson and Nowell, 1970; Lafferty and Jones, 1969), several groups, using various culture conditions have established that in some species combinations MLR reactivity to xenogeneic cells is of the same magnitude as that to allogeneic cells (Widmer and Bach, 1972; Shons *et al.*, 1973; Asantila *et al.*, 1974).

Data from Asantila's group suggested that the degree of responsiveness in xenogeneic combinations was a function of the phylogenetic distance between the two reacting species. Thus, while the response of human lymphocytes to rat lymphocytes was strong, the response to frog lymphocytes was considerably weaker (Asantila *et al.*, 1974).

Recently, Lindahl and Bach (1976), in a human/mouse system, demonstrated a significant connection between the antigens stimulating in allogeneic MLR and those stimulating in xenogeneic MLR. Accordingly, I region coded antigens caused a stronger proliferative response than either H-2K or H-2D region coded antigens.

Early studies by Berke *et al.*(1971) in a rat/mouse system indicated that cytotoxic cells were also produced in xenogeneic MLR. Although these cells were shown to be specific for the sensitising strain they were not identified as T cells and their reactivity was not compared to similarly activated allogeneic cells.

More recent data from *in vivo* studies in a rat/mouse system (Hines *et al.*, 1976) and *in vitro* studies in a human/mouse system (Lindahl and

Bach, 1976) have established that xenoantigens do stimulate a CML response, but in contrast to the MLR data, this response is considerably weaker than that generated in an allogeneic interaction. Utilising a restimulation technique to analyse the mouse specificities that stimulate human cytotoxic T cell precursors, Lindahl and Bach (1975, 1976) concluded that in xenogeneic, as well as in allogeneic combinations, cytotoxicity is directed predominantly against antigens coded by the H-2K and H-2D regions of the MHC.

Although these results are preliminary, they suggest that the antigens recognised by human lymphocytes may well be identical to those recognised by mouse lymphocytes in the mouse allogeneic reaction. The generally depressed xeno CML responses indicate that in any animal there are more cytotoxic T cell precursors with specificity for alloantigens than for xenoantigens. Possible explanations for these differences will be considered in a later chapter.

(b) It has been well documented that when cytotoxic T cell precursors are sensitised with syngeneic, virus-infected lymphoid cells (Zinkernagel and Doherty, 1974a; Blanden *et al.*, 1976; Koszinowski and Ertl, 1975), TNP-treated lymphoid cells (Shearer, 1974; Forman, 1975; Koren *et al.*, 1975) or lymphoid cells bearing markers absent on the responding population such as minor histocompatibility antigens (Bevan, 1975; Gordon and Simpson, 1975), the resultant cytotoxic effector population can only recognise and lyse targets which share its H-2K or H-2D haplotype. This phenomenon has been termed "H-2 restriction" and was concurrently described by Zinkernagel and Doherty (1974a) in the LCM virus system and Shearer (1974) in the TNP system.

Despite the overwhelming evidence for "H-2 restriction" at the effector level, such restriction is not observed at the induction level. This point has been well illustrated in experiments using radiation chimaeras (Zinkernagel, 1976; von Boehmer and Haas, 1976) where it has

been shown that the cytotoxic T cell precursor can "see" antigen associated with any H-2K or H-2D haplotype but once sensitised to this combination it will only lyse targets presenting the same configuration. In effect then, "H-2 restriction" occurs at the level of the stimulator and the target rather than the effector and the target.

The involvement of the MHC in the induction and effector phases of the immune response is not limited to cytotoxic T cells. There is also some evidence that T helper cells recognise antigen in association with Ia antigens (Erb and Feldman, 1975a) while T cells responsible for DTH see some antigens (e.g., fowl gamma globulin) in association with Ia antigens and others (e.g., DNFB) in association with H-2K or H-2I gene products (Miller *et al.*, 1975, 1976). Thus, H-2K (or D) cell surface moieties themselves (or other products of closely linked genes coding in the MHC locus) and the I region coded Ia antigens are intimately involved in antigen recognition by various subsets of T cells, i.e., H-2 appears to be *a priori* for T cell recognition.

With respect to cytotoxic T cells this involvement may be explained by one of at least three mechanisms:

1. physiological interaction
2. altered self
3. Ag-H-2 complexing.

The feasibility and universality of each of these mechanisms has been discussed at length in numerous reviews (Doherty *et al.*, 1976; Blanden *et al.*, 1976; Forman *et al.*, 1976; Shearer *et al.*, 1976) and will only be briefly considered here.

The physiological interaction model as originally described by Zinkernagel and Doherty (1974b) has been generally discredited experimentally and will therefore not be further discussed (Zinkernagel, 1976; van Boehmer and Haas, 1976; Davidson *et al.*, 1976).

Two mechanisms for altered self have been proposed. The first, which was originally suggested by Zinkernagel and Doherty (1974b) as an alternative to physiological interaction requires that viruses act as modifying agents which immunologically alter cell surface structures coded by the MHC. Although chemical alteration may be feasible in the case of viruses, as a more general explanation this mechanism is deficient, particularly if the data from studies with minor histocompatibility antigens is considered.

The second mechanism for altered self requires that non-H-2 antigens become closely associated with H-2K or H-2D region controlled products on the cell membrane with the resultant creation of new antigenic determinants (NAD's) or interaction antigens (Bevan, 1975) which may have components provided by both H-2 and antigen.

The last mechanism, antigen H-2 complexing, involves the physical association of antigen and cell surface MHC products to form an antigen complex in which both components retain their antigenic integrity.

The last two mechanisms are currently the most favoured as explanations for the apparent requirement for H-2 recognition for T cell induction. It should be emphasised, however, that both of these models should be regarded only as working hypotheses as our present understanding of events occurring at the molecular level is, to say the least, rudimentary.

From the foregoing it is clear that cytotoxic T cells recognise something special about H-2K (or D) region coded determinants. Whatever the nature of the receptors involved, their dictionary appears to extend from self to xeno histocompatibility antigens. In the syngeneic systems described, H-2K or H-2D coded structures are possibly recognised in either physical or chemical association with other membrane expressed antigens. The fundamental question therefore arises whether a universal recognition system exists such that allogeneic and xenogeneic systems

also involve recognition of structures other than, or in addition to, H-2K or H-2 coded antigens or whether T cells with completely different receptor dictionaries are involved.

1.7 The nature of the cytotoxic T cell receptor for antigen

While it is clear that membrane bound immunoglobulin molecules serve as receptors for antigen on B cells, the nature of the antigen specific receptor on T cells has remained elusive. There are three potential classes of receptors:

1. Immunoglobulin (Ig)
2. Structures consisting of Ig and non-Ig subunits
3. Non-Ig receptors.

There has been considerable controversy in the past over the presence of conventional Ig classes on T cell surfaces (Crone *et al.*, 1972; Marchalonis and Cone, 1973). However, in the past few years evidence has been accumulating which suggests that normal immunocompetent B and T lymphocytes with the capacity to react against the same antigenic determinants share idiotypic determinants (Ramseier and Lindenmann, 1972; McKearn, 1974; Eichmann and Rajewsky, 1975; Binz and Wigzell, 1975a). The general conclusion drawn from these studies is that B and T lymphocytes reacting to the same antigenic determinants use the same set of V genes to code for the relevant antigen binding receptors. The findings of other workers (Eichmann and Rajewsky, 1975; Black *et al.*, 1976 and Hämmerling *et al.*, 1976), using anti-idiotypic antibody to stimulate idiotype positive T cells are also consistent with this view. Genetic linkage experiments in the latter system indicate that the T cell idiotypic determinants are coded by genes linked to the heavy chain allotypes of Ig molecules. Recently, Binz and Wigzell (1976) have shown that T cells release, in soluble form, molecules carrying antigen binding specificity as well as idiotypic markers. These molecules exist as monomers of molecular

weight approximately 70,000 daltons or as dimers of molecular weight 150,000 daltons. There is no evidence that either of these T cell moieties is linked to conventional light chain molecules. Similarly, conventional serological markers of the constant region of heavy or light chains were not in evidence either.

Thus, at the moment, there is considerable evidence to suggest that at least part of the T cell receptor system consists of a single or dimeric Ig H chain having a previously undescribed constant region.

Studies examining the specificity of T cell recognition in binding and functional assays indicate that the ability to discriminate between related but different synthetic antigens is at least as great as that found with the B cell (Schlossman *et al.*, 1969; Hämmerling and McDevitt, 1974; Janeway, 1976). The question then arises whether T cells with a receptor system composed solely of Ig chains could allow such fine discrimination to occur. Although it is known that Ig H chains bind antigen specifically, albeit with lower affinity than whole Ig H (Utsumi and Kanush, 1964), little is known about the fine specificity of Ig H chains or their binding properties with complex antigens.

If, in fact, T cells do recognise MHC coded determinants and membrane bound antigens as independent moieties on the cell surface, it is highly probable that they use a different gene product for each receptor unit. Thus, just as B cells make two genetically unlinked H and L chains, T cells may also make genetically independent receptor structures which may or may not be linked on the cell surface.

Given that two independent receptors do exist on T cells, the second, and as yet undefined, receptor must be largely confined to recognising self and foreign MHC antigens. Similarly, at least some of the V_H gene coded receptors must also be involved in MHC coded determinant recognition as well in order to account for the large number of T cells reported to respond to alloantigens (Wilson *et al.*, 1968; Ford *et al.*, 1975; Binz and Wigzell, 1975b; Martz, 1975).

From the foregoing brief outline of the dictionary range and possible nature of the receptors on cytotoxic T cells it is obvious that, despite intensive investigations, the exact nature of the T cell receptor system and the type of antigens it recognises is still a matter of speculation.

1.8 Nature of the stimulator population

Although it is established that both the recognitive and effector phases of MLR are mediated by T cells the tissue distribution of potential stimulator populations remains unclear.

Until recently, most attempts to ascertain the cell types with stimulating capacity have been concerned primarily with measurement of proliferative rather than cytotoxic responses. The data summarised in Table 1.2 gives an indication of some of the cell types which have been tested.

Although many of the reports are conflicting it appears that allogeneic normal or transformed lymphoid populations generally stimulate better than non-lymphoid populations. This holds true for both proliferative and CML responses in all of the animal species tested so far. Some exceptions do exist, however, in that epithelium, endothelium and spermatazoa have also been reported to cause significant proliferation, and in one instance, epithelial cells generated cytotoxic activity as well. Whether the stimulation observed with non-lymphoid populations is the result of Ia antigens present on their surfaces (Hämmerling *et al.*, 1975), or alternately reflects contamination of the cell preparations with small but significant numbers of lymphoid cells is, as yet, unresolved.

The discrepancies observed in the levels of the proliferative and cytotoxic T cell responses generated following stimulation with B cells, T cells or macrophages may relate to differences in MLC methodology, mouse strains or cell purification techniques.

TABLE 1.2

THE STIMULATOR POTENTIAL OF VARIOUS LYMPHOID AND NON-LYMPHOID POPULATIONS

Cells	Donor	MLR	CML	Authors
A. Lymphoid	Mouse	+++	NT	Cheers and Sprent, 1973
T	Mouse	NT	+++	Wagner and Wyss, 1973
	Mouse	+	NT	Plate and McKenzie, 1973
	Mouse	+++	NT	von Boehmer, 1974
	Mouse	+++	NT	Lonai and McDevitt, 1974
	Mouse	-	-	Simpson, 1975
	Human	-	NT	Lohrmann *et al.*, 1974
	Human	+++	+++	Sondel *et al.*, 1975
B	Mouse	+++	NT	Cheers and Sprent, 1973
	Mouse	NT	+++	Wagner and Wyss, 1973
	Mouse	+++	NT	Plate and McKenzie, 1973
	Mouse	+++	NT	von Boehmer, 1974
	Mouse	+++	NT	Lonai and McDevitt, 1974
	Mouse	+++	+++	Simpson, 1975
	Human	+++	NT	Lohrmann *et al.*, 1974
	Human	+++	+++	Sondel *et al.*, 1975
Macrophages	Human	++	NT	Levis and Robbins, 1970
	Human	+++	NT	Bain and Lowenstein, 1969
	Human	++	NT	Alter and Bach, 1970
	Human	+++	NT	Rode and Gordon, 1973
	Human	+++	NT	Twomey *et al.*, 1970
	Mouse	+++	NT	Talmage and Hemmingsen, 1975
	Mouse	+++	NT	Schirrmacher *et al.*, 1975

Table 1.2 (contd.) -

Cells	Donor	MLR	CML	Authors
Macrophages (contd.)	Mouse	+++	+++	Simpson, 1975
	Mouse	NT	+	Wagner, 1973
	Guinea pig	+++	NT	Greineder and Rosenthal, 1975
B. Neoplastic cell lines				
Myeloma	Mouse	NT	+++	Wagner and Wyss, 1973
Thymoma	Mouse	NT	+++	Wagner and Wyss, 1973
T lymphoblast	Human	-	-	Royston *et al.*, 1974
		+++	+++	Callewaert *et al.*, 1975
B lymphoblast	Human	+++	+++	Royston *et al.*, 1974
		+++	+++	Callewaert *et al.*, 1975
HeLa	Human	-	NT	Hardy and Ling, 1969
Lymphoma	Human	+++	NT	Hardy and Ling, 1969
L_2C leukaemia cells	Guinea pig	-	NT	Greineder and Rosenthal, 1975
C. Non-lymphoid				
Polymorpho nuclear cells	Human	-	NT	Rode and Gordon, 1973
	Human	-	NT	Mardiney *et al.*, 1972
	Guinea pig	-	NT	Greineder and Rosenthal, 1975
Platelets	Human	-	NT	Rode and Gordon, 1974
Epithelium	Mouse	+++	NT	Gillette *et al.*, 1972
	Mouse	NT	+++	Steinmuller and Wunderlich
	Rat	+++	NT	Main *et al.*, 1971
	Rat	+++	NT	Lane and Ling, 1973

Table 1.2 (contd.) -

Cells	Donor	MLR	CML	Authors
Epithelium (contd.)	Human	+++	NT	Lane *et al.*, 1975
		++	NT	Levis and Miller, 1972
		++	++	Hirschberg *et al.*, 1975
Endothelium	Dog	+++	NT	Vetto and Burger, 1972
	Human	+++	NT	Hirschberg *et al.*, 1975
Sperm	Human	+++	NT	Levis *et al.*, 1976
Brain, kidney thyroid	Rat	-	NT	Lane and Ling, 1973
Melanoma, Osteosarcoma	Human	-	NT	Han, 1972
Fibroblasts	Mouse	+	+	Wagner and Wyss, 1973
	Rat	NT	+++	Berke ., 1969
				Lonai ., 1972
	Human	-	NT	Schellekens and Eijsvoogel, 1970
	Guinea pig	-	NT	Greineder and Rosenthal, 1975
RBC	Human	-	NT	Hardy and Ling, 1969

NT - not tested

+++ - good response

++ - moderate response

+ - poor response

- - no response

In addition to being lymphoid stimulator cells must also be physically intact and metabolically active. Thus, sub-cellular alloantigen preparations stimulate weak or negligible primary proliferative or cytotoxic responses (Gutterman *et al.*, 1972; Wagner and Boyle, 1972; Engers *et al.*, 1975b; Häyry and Andersson, 1976; Wagner *et al.*, 1976a). Similarly, actinomycin A or D treatment (Wagner, 1973) heat treatment (Eijsvoogel *et al.*, 1973; Schendel and Bach, 1974; Wagner, 1973), UV irradiation (Lafferty *et al.*, 1974; Wagner *et al.*, 1974, Schendel and Bach, 1975; Häyry and Andersson, 1976; Röllinghoff and Wagner, 1975) or fixation (Dennert and Lennox, 1974) of the stimulator population abrogates stimulator capacity. In contrast to the requirements for primary stimulation, those for secondary stimulation are much less stringent. Accordingly, sub-cellular fractions of tumour or lymphoid cells (Engers *et al.*, 1975b; Häyry and Andersson, 1976; Wagner *et al.*, 1976a) and UV treated (Häyry and Andersson, 1976; Röllinghoff and Wagner, 1976), or fixed (Dennert and Lennox, 1974), lymphoid or tumour cells are all capable of restimulating previously primed populations to the same extent as untreated cells.

To summarise, the stimulator cell, at least in primary induction, appears to be more than an inert vehicle of transplantation antigens and may in fact, as suggested by Lafferty and Cunningham (1975), actively participate in the stimulation process. Although subcellular fractions stimulate poorly in primary cultures and do not inhibit the activity of cytotoxic T cells against allogeneic target cells (Engers *et al.*, 1975b) it remains to be determined whether cytotoxic T cell precursors respond to secreted transplantation antigens or only to membrane bound antigens. The requirement for macrophages for the generation of cytotoxic T cells demonstrated by Wagner and colleagues (1972) has been taken by these workers as an indication that soluble antigens may be the stimulating moieties (Wagner, 1973; Wagner *et al.*, 1972).

Several models, based on the Bretscher and Cohn 2-signal model for B cell induction (Bretscher and Cohn, 1970) have been proposed to explain the induction of cytotoxic T cell responses. These will be discussed in detail in subsequent chapters.

1.9 Mechanisms of cytotoxicity

The process by which a T cell is able to lyse a target cell has been shown, by the use of various pharmacologic agents and incubation at different temperatures, to be a multi-staged event (Berke and Amos, 1973b; Henney, 1973; Wagner and Röllinghoff, 1974).

It is generally agreed that the first stage involves the physical adhesion between the effector cell and target cell (Brondz, 1968; Golstein *et al.*, 1971; Berke and Levey, 1972). This is rapidly followed by the second, post-recognition stage where a "lethal hit" of an unknown nature is delivered to the target cell (Wagner and Röllinghoff, 1974; Martz, 1975). The final, target cell disintegration stage results in the release of radioisotope from the pre-labelled target cells. In contrast to the first two stages, the final stage does not require the continual presence of an active effector cell population (Martz and Benacerraf, 1973; Miller and Dunkley, 1974; Wagner and Röllinghoff, 1974).

The chelating agents EDTA and EGTA have proven useful in distinguishing the various stages of cytolysis. Early studies by Mauel *et al.* (1970) showed that if EDTA was added at the initiation of an *in vitro* cytotoxicity assay, no specific ^{51}Cr release was seen. However, if the addition was delayed by 45 minutes, the rate of ^{51}Cr release proceeded normally. Henney and Bubbers (1973) confirmed and extended these observations and concluded that the EDTA effect was reversible and blocked some lytic process requiring Ca^{++} and/or Mg^{++}.

Stulting and Berke (1973) established that the initial recognitive phase, as investigated by specific absorption on monolayers, can occur in the presence of either Ca^{++} or Mg^{++}. This work has subsequently been

confirmed by Golstein and Smith (1976) in a similar system. In contrast, Plaut *et al.* (1976) have shown the first stage to be more Mg^{++} than Ca^{++} dependent.

The temperature dependency of the first stage is controversial. Thus Goldsein *et al.* (1971) found that adherence of effector populations to monolayers occurred at 37°C, but not at 4°C, whereas Stulting and Berke (1973), using a similar technique, found that significant adherence occurred at 25°C. Martz (1975) has shown that adhesions between centrifuged killers and targets occur at 15°C, but are optimal at 37°C. In contrast, Wagner and Röllinghoff (1974), using a similar centrifugation technique, concluded that the recognition phase could occur at 4°C. However, since adhesion between centrifuged cells may occur in one minute at 37°C (Martz, 1975), the warming period between 4°C and 45°C which the cells must pass through in Wagner's system in order to be inactivated may be of sufficient duration for both adhesion and lethal hit to occur. The first phase therefore appears to be relatively temperature independent with optimal binding occurring at 37°C.

The binding of killer to target has been shown to be reversible in the very early stages of phase I (Wagner and Röllinghoff, 1974; Martz, 1975). Binding is also energy dependent (Werkele *et al.*, 1972) and completely, but reversibly, inhibited by cytochalasin B (Cerottini and Brunner, 1972; Henney, 1973). It is not known whether the action of cytochalasin B is at the level of disruption of microfilaments or membrane transport (for review, see Cerottini and Brunner, 1974).

The second, or post-recognition-hit stage of cytolysis required Ca^{++} rather than Mg^{++} (Golstein and Smith, 1976; Plaut *et al.*, 1976) and in contrast to the first stage is highly remperature dependent (Berke *et al.*, 1972b; Wagner and Röllinghoff, 1974) and irreversible (Wagner and Röllinghoff, 1974). The second stage is inhibited by colchicine and adenyl cyclase activators such as the β catecholamines and cholera

enterotoxin, but is unaffected by cytochalasin B (for review, see Henney, 1973). Although the first two phases of the lytic cycle appear to be separable by several criteria it is notable that target cells become committed to lysis within minutes of contact with killer cells, so the duration of both phases is extremely short (Wagner and Röllinghoff, 1974; MacDonald, 1975; Martz, 1975).

The final stage in the lytic cycle is Ca^{++} and Mg^{++} independent (Henney and Bubbers, 1973; Martz, 1975; MacDonald, 1975); is highly temperature dependent (Wagner and Röllinghoff, 1974); occurs independently of the presence of killer cells (Martz and Benacerraf, 1973; Wagner and Röllinghoff, 1974; Miller and Dunkley, 1974) and in contrast to the first two stages requires three-four hours in order to be completed (Wagner and Röllinghoff, 1974; Martz and Benacerraf, 1973).

Recent kinetic studies with limiting numbers of effectors or labelled effectors have indicated that the sensitized cytotoxic T cell is unharmed during its interaction with a specific allogeneic target. Further, after administering lethal damage to one target cell, the killer cell retains its capacity to destroy other target cells (Brunner *et al.*, 1970; Berke *et al.*, 1972b; Andersson and Häyry, 1973; Martz, 1976). Data from Martz' laboratory suggest that a single killer cell may be able to sequentially kill more than six individual target cells (Martz, 1976). Hence, even a small number of specifically sensitised cytotoxic T cells could play an important role in the destruction of "foreign" cells *in vivo*.

The mechanism by which cytotoxic T cells inflict their "lethal hit" remains a mystery. Berke and Amos (1973b) and Martz (1976) have suggested that killer cells cause permeability lesions in target cell membranes, possibly by alteration of transmembrane potential, which ultimately kill the cell, probably by a combination of loss of intracellular metabolite pools and colloid osmotic swelling. Alternately, Henney (1973) has postulated that killing results from the action of a secreted antigen-

induced mediator or toxin. The latter alternative seems unlikely for several reasons. Firstly, if such a toxin did exist, it would have to be highly specific and have a short range of action, as no toxic substances have been detected in supernatants from cytotoxicity assays (Häyry *et al.*, 1972; Martz and Benacerraf, 1973), and third partly labelled bystander targets and the effector population itself are not destroyed (Cerottini and Brunner, 1974). Secondly, in the event of a non-specific toxin, one would have to postulate that the effector cell had evolved resistance to its own toxic product, since cytotoxic T cells have been shown to be susceptible to killing by other specifically induced cytotoxic T cells (Golstein, 1976; Martz, 1976). Finally, the toxin would have to be non-protein in nature since protein synthesis inhibitors such as pactamycin and emetine reportedly do not affect lytic activity (Thorn and Henney, 1976).

CHAPTER 2

QUANTITATION OF THE CYTOTOXIC RESPONSE

2.1 Introduction

With the possible exception of a plaquing assay recently described by Bonavida and colleagues (1976) there are no methods yet available for the precise estimation of the frequency of cytotoxic T cells or their precursors. However, kinetic studies using the ^{51}Cr release assay described by Brunner *et al.* (1968), have led to the formulation of mathematical models which estimate the relative cytotoxic activity of effector populations.

Although release of ^{51}Cr is a sensitive measure of target cell damage, it is not *per se* a quantitative assay of effector cell activity. For comparison of the relative number of cytotoxic effector cells in different populations, it is necessary to establish a quantitative relationship between the cytotoxic activity (i.e., percentage of chromium released) and the number of effector cells present.

Canty *et al.* (1971), and later MacDonald *et al.* (1973a), observed that percentage of target lysis plotted as a function of the logarithm of the sensitized cell concentration generated a sigmoid dose response curve. The midportion of this curve was approximately linear and its slope independent of the number of cells present. By determining the number of lymphoid cells required to obtain a fixed lysis value on the linear portion of the curve, MacDonald *et al.* (1973a) were able to obtain a quantitative estimate of the relative frequency of effector of progenitor cells in the population. Cerottini and Brunner (1971) used a similar analysis to define a lytic unit which is the number of effector cells required to produce 33 per cent lysis of 25,000 target cells. Both of these methods make possible quantitative comparisons of the cytolytic activities of different lymphoid populations. Henney (1971) observed that the specific lysis curves obtained in the ^{51}Cr release assay could be

reasonably well fitted by an expression of the form:

$$y = 1 - e^{-KN}$$

where, K = constant

N = total number of sensitised cells

y = fractional specific lysis.

This expression is identical to that predicted by the Poisson probability distribution for the fraction of cells inactivated by a discrete process in which "one hit" is sufficient for inactivation (Zimmer, 1961). In the same report, Henney was able to show that when specific lysis was plotted on a log/log scale against the number of effector cells the slope obtained approximated one, thus implying that cell-mediated lysis is a "one hit" phenomenon. Similar findings have also been reported by Lafferty *et al.* (1974a). Together, these studies confirmed earlier work with cytotoxic rat lymphocytes by Wilson (1965) and Berke *et al.* (1969) which also suggested that a "single hit" was sufficient for target cell inactivation.

Miller and Dunkley (1974) have given a theoretical basis for Henney's observations and have shown that the interaction between cytotoxic cells and targets can be described by:

$$\alpha = \frac{-\ln(1 - p)}{Nt}$$

where, α is proportional to the frequency of cytotoxic T cells

N = the number of lymphoid cells used in the assay

t = incubation time

p = fractional specific lysis.

Alpha (α) can be evaluated from any single value of lysis where fairly dilute suspensions of target cells and lymphocytes are assayed.

The cytotoxic activity of the lymphoid populations described in the following chapters will also be expressed in terms of a derived unit, namely the cytotoxic unit. This chapter will primarily be concerned with the mathematical derivation of the cytotoxic unit and a description of the

experiments designed to determine the optimum conditions for the generation of cytotoxic T cells *in vitro*.

2.2 Materials and methods

2.2.1 Animals

8 - 10 week old CBA/H and BALB/c inbred mice maintained in the Animal Breeding Establishment of this school were used.

2.2.2 Tissue culture cell lines

The DBA/2 mastocytoma cell line, P815-X2 was used as the target in cytotoxicity assays. The cell line was maintained *in vitro* in 75 cm^2 Falcon tissue culture flasks (Falcon Plastics, Oxnard, California, USA) containing 30 ml of Dulbecco's Modified Eagle's Medium (Cat. No. H-16, GIBCO, Grand Island, NY, USA) supplemented with 10% heat inactivated (56°C for 30') foetal calf serum (FCS, CSL, Melbourne, Victoria) and antibiotics (100 µg/ml streptomycin and 100 units/ml penicillin G). The flasks were gassed with 10% CO_2, 7% O_2, and 83% N_2 and maintained at 37°C.

Cells were cultured at an initial concentration of 10^5 viable cells/ml. Subcultures were made at 2-3 day intervals when the cell concentration was 1-2 x 10^6 viable cells/ml. Cells for use as targets were generally obtained by subculturing cells from 2-3 day cultures overnight in fresh medium.

2.2.3 Preparation of lymphoid cell suspensions

Spleens and lymph nodes (mesenteric, popliteal, lumbar, and inguinal) were collected aseptically, cut into small pieces and pressed through a fine stainless steel sieve into Eagle's Minimal Essential Medium (Cat. No. F-15, GIBCO, Grand Island, NY, USA) containing antibiotics (100 µg/ml streptomycin and 100 units/ml penicillin G), 10^{-4}M 2-mercaptoethanol and 10% heat inactivated foetal calf serum (CSL, Melbourne). This medium will henceforth be referred to as "culture medium". Cell suspensions were washed and declumped three times. Cell viability was determined by Trypan blue exclusion and was usually better than 90%.

Stimulator cell populations were adjusted to 4 x 10^6/ml, gassed with a mixture of 10% CO_2, 7% O_2, and 83% N_2 and incubated for 90 minutes at 37°C. These cells were then irradiated with 1000 R of gamma irradiation from a cobalt-60 source.

2.2.4 Mixed lymphocyte cultures

Mixed lymphocyte cultures were set up according to the method of Lafferty *et al.* (1974a,b) with some modifications. Briefly, 1.0 ml of the stimulator cell suspension and 1.0 ml of the responder cell suspension were mixed in 16 mm diameter wells in 24-well trays (Linbro Model FB-16-24-TC Multidish Disposo-trays, Linbro Chemical Co., New Haven, Conn., USA). All assays were set up in triplicate. Trays were placed in air-tight boxes, gassed with humidified 10% CO_2, 7% O_2, 83% N_2 and maintained at 37°C for 4-7 days. Except where otherwise stated stimulator populations were BALB/c spleen cells and responder cell populations were CBA/H lymph node cells.

2.2.5 Cytotoxicity assay

P815 cells were harvested from culture flasks and washed three times with H-16. For labelling, the cell concentration was adjusted to 5 x 10^6 viable cells/ml and 100 µCi/ml of ^{51}Cr (Na_2 ^{51}Cr O_4) was added. Tubes were gassed with 10% CO_2, 7% O_2, 83% N_2 and incubated for 60' at 37°C. Cells were then washed three times in F-15 + 10% HIFCS and made up to a final concentration of 1 x 10^6 viable cells/ml.

Cytotoxic cell populations consisted of cells pooled from triplicate culture wells which were centrifuged once and resuspended in 2.0 ml of culture medium. Twofold serial dilutions were made over a 0-8fold range. 0.1 ml of each dilution was added to 0.1 ml of labelled target cells in 96 well 6 mm diameter culture trays (Linbro model IS-FB-96-TC Multidish Disposo trays). All assays were set up in quadruplicate. Spontaneous ^{51}Cr release was determined by adding 0.1 ml of culture medium to 6 wells containing 0.1 ml of target cells, while total releasable ^{51}Cr was determined by adding 0.1 ml of target cells to tubes containing 0.9 ml of

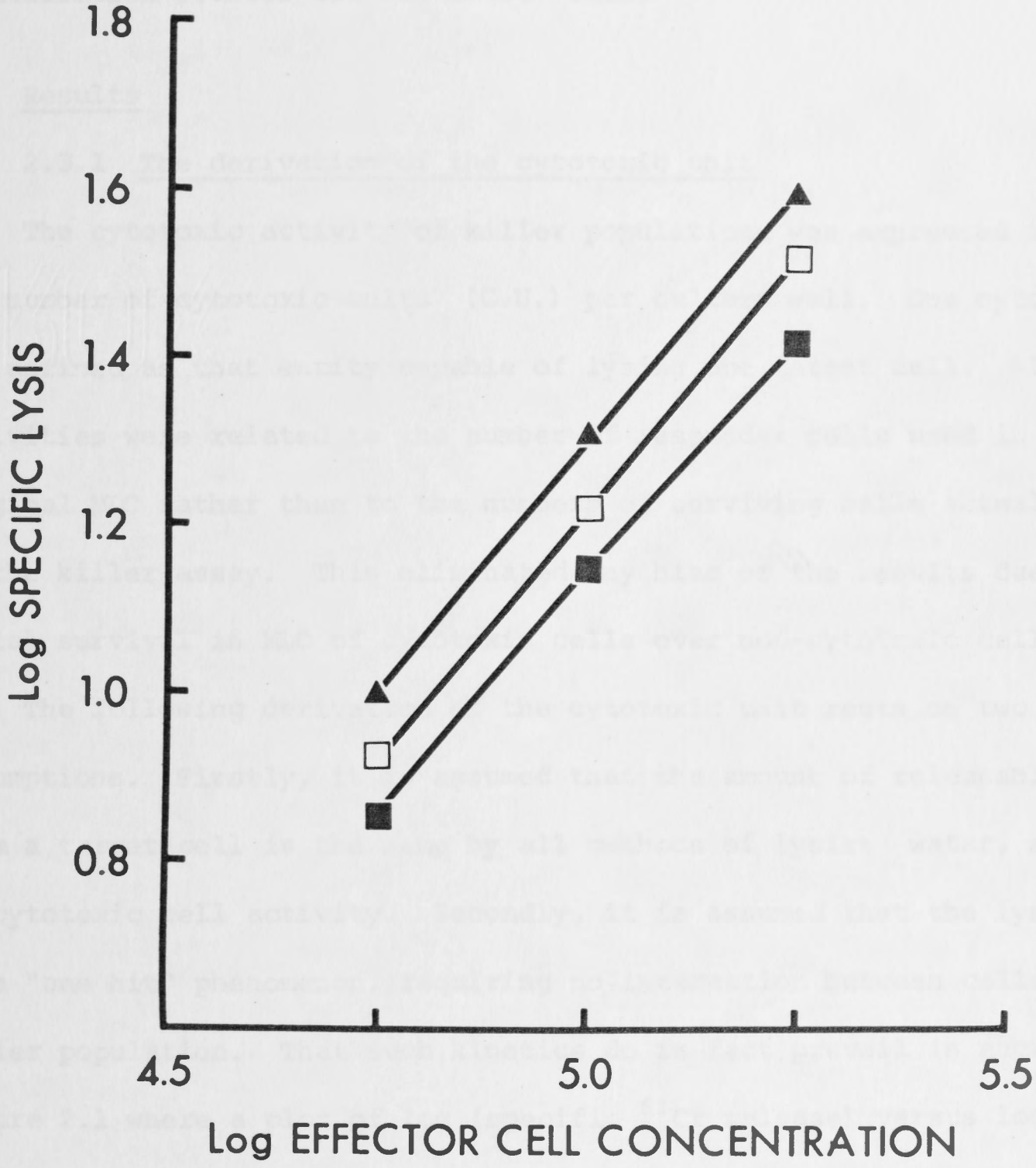

Figure 2.1 - Log/log plot of specific lysis vs. effector cell concentration indicates linear relationship with slope ∿1.0, implying lysis is a 'one-hit' phenomenon. 4 x 10^6 BALB/c stimulator cells were cultured with 2 x 10^6 CBA/H lymph node responder cells and assayed on day 5 on ^{51}Cr labelled P815 targets. ▲, □, ■, represent three separate experiments.

distilled water. All mixtures were incubated for 4 hours at 37°C. Aliquots, 0.5 ml of the supernatants of water lysis controls and 0.1 ml of the supernatants of all other samples, were taken and counted in a Packard Gamma Scintillation Counter for one minute each.

2.3 Results

2.3.1 The derivation of the cytotoxic unit

The cytotoxic activity of killer populations was expressed in terms of the number of cytotoxic units (C.U.) per culture well. One cytotoxic unit was defined as that entity capable of lysing one target cell. All cytotoxic activities were related to the number of responder cells used in the original MLC rather than to the numbers of surviving cells actually used in the killer assay. This eliminated any bias of the results due to preferential survival in MLC of cytotoxic cells over non-cytotoxic cells.

The following derivation of the cytotoxic unit rests on two basic assumptions. Firstly, it is assumed that the amount of releasable ^{51}Cr from a target cell is the same by all methods of lysis: water, spontaneous or cytotoxic cell activity. Secondly, it is assumed that the lysis process is a "one hit" phenomenon, requiring no interaction between cells in the killer population. That such kinetics do in fact prevail is shown in Figure 2.1 where a plot of log (specific ^{51}Cr release) versus log (No. of effector cells) resulted in a straight line with a slope approximating one. Since the killer-target interaction is "one hit", the amount of chromium released in unit time will be proportional to:

(a) the number of targets one killer can contact in unit time,

(b) the proportion of live contacts which cause lysis,

(c) the number of killer cells present,

(d) the proportion of live targets remaining in the culture.

Now if we define -

N = No. of targets one killer can contact in unit time

μ = proportion of live contacts which cause lysis

t = duration of the assay

y_o = total number of target cells

y_c = number of targets lysed by a number, c, of the killer population

α = ^{51}Cr release by one lysed target

then

αy_o = total specifically releasable ^{51}Cr

αy_c = ^{51}Cr specifically released by c cells of killer population

and

$\frac{y_o - y_c}{y_o}$ = proportion of killer-target interactions involving live targets,

then, the number of targets killed in unit time is proportional to:

$$C.N.\mu \left| \frac{y_o - y_c}{y_o} \right|$$

i.e.

$$\frac{d(y_o - y_c)}{dt} = -M.c.N.\mu \left| \frac{y_o - y_c}{y_o} \right|$$

where M is a constant equal to the fraction of the killer population which are active killers.

$$\therefore \quad y_o - y_c = Ae^{-(M.c.N.\mu.t/y_o)}$$

when $c=0$, $y=0$, $\therefore A = y_o$

also, $M.c.N.\mu.t$ = total number of targets lysed by c cells of killer population

= number of cytotoxic units in c cells of killer population

= U_c (say)

$$\therefore \quad y_o - y_c = y_o e^{-U_c/y_o}$$

$$\therefore \quad 1 - \frac{y_c}{y_o} = e^{-U_c/y_o}$$

Now,

$$\frac{y_c}{y_o} = \frac{\alpha y_c}{\alpha y_o}$$

$$= \frac{\text{specific } {}^{51}\text{Cr release by c cells of killer populn.}}{\text{total specifically releasable } {}^{51}\text{Cr}}$$

$$= y \quad \text{(say)}$$

$$\therefore \quad 1 - y = e^{-U_c/y_o}$$

$$\therefore \quad \ln(1 - y) = -\frac{U_c}{y_o}$$

$$\therefore \quad U_c = -y_o \ln(1 - y)$$

$$= \text{C.U. /assay}$$

$$\therefore \quad \text{Number of C.U. per MLC culture} = \frac{-y_o \ln(1 - y)}{f}$$

where f is the fraction of the culture assayed.

2.3.2 Expression of results

All estimations of cytotoxic activity will be expressed as log C.U./culture. The values reported are means of values obtained with three different effector to target cell ratios.

Conventionally, cytotoxicity data is expressed as a value $\pm$ 2 S.E. where the standard error is calculated from replicates of a single population of effectors. Since this is not a true estimation of the variability a different approach has been adopted here. The variability was calculated by assaying twenty individual cultures at the peak of the response and calculating the S.D. from the values obtained. This estimation has been carried out on several occasions in this and other laboratories (K. Lafferty, personal communication) and a S.D. value of

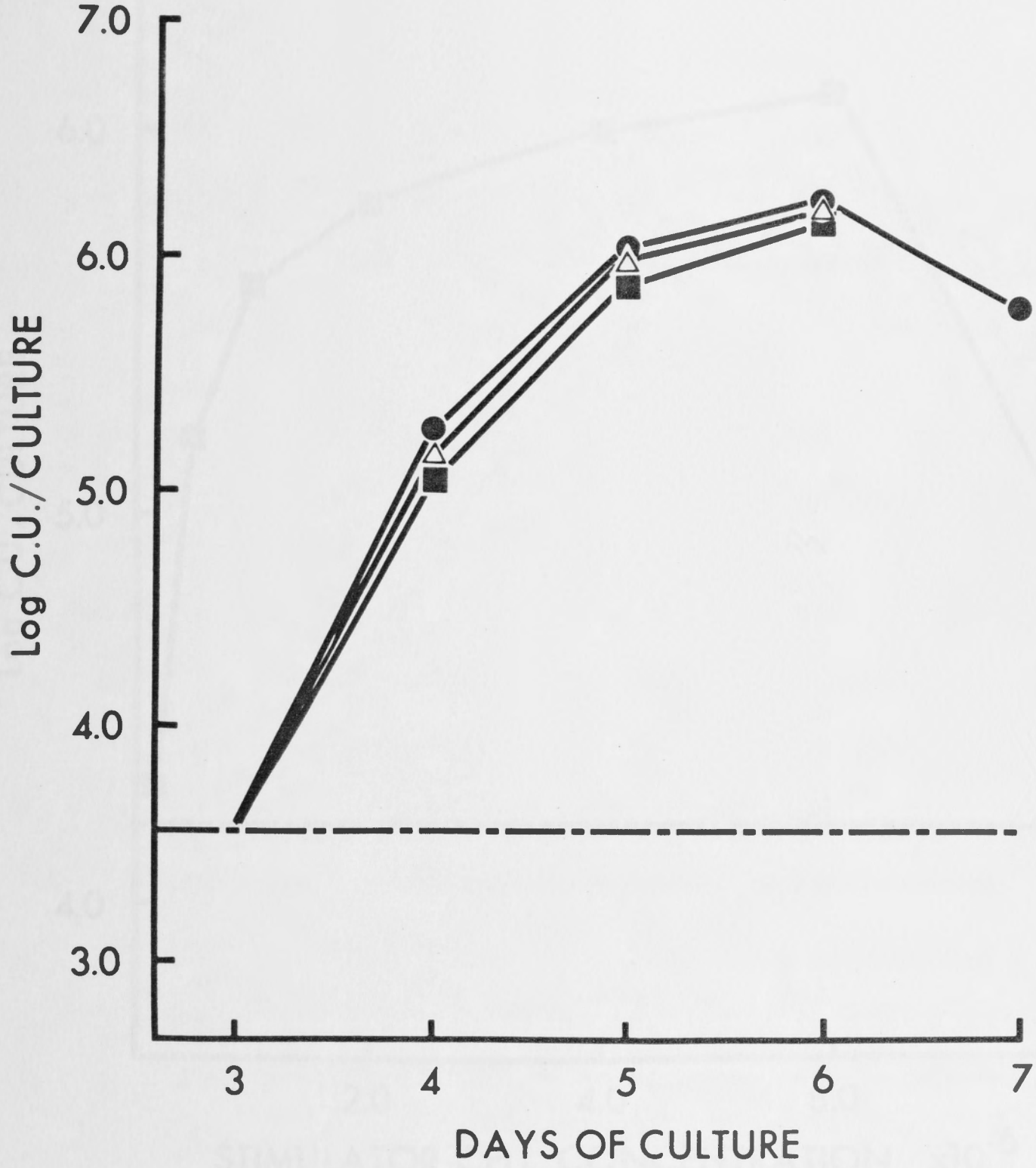

Figure 2.2 - Time course for the generation of cytotoxic T cell activity *in vitro*. ● , 4 x 10^6; △ , 8 x 10^6; ■ , 2 x 10^6 BALB/c stimulator spleen cells were cultured with 2 x 10^6 CBA/H lymph node responder cells for the time intervals indicated. Cultures were assayed on ^{51}Cr labelled P815 cells. ▬ ▬ ▬ , background.

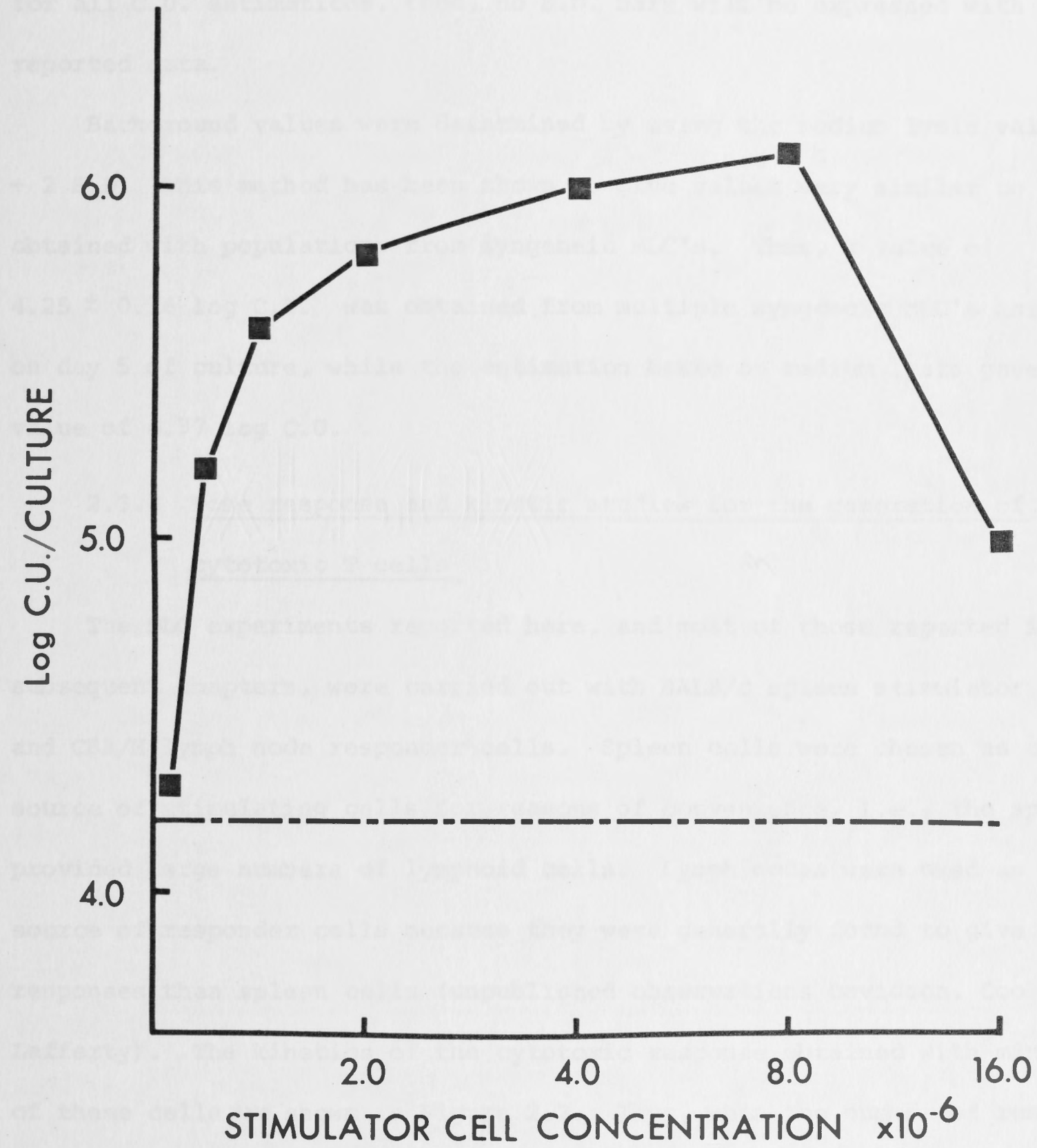

Figure 2.3 - Dose response curve obtained when 2 x 10^6 CBA/H lymph node cells were cultured with varying numbers of BALB/c spleen stimulator cells. All cultures were assayed on day 5 on 1 x 10^5 ^{51}Cr labelled P815 target cells. ▬ ▪ ▬ , background.

0.06 log C.U. has consistently been obtained. A value of ± 2 S.D., i.e. 0.12 log C.U. has therefore been arbitrarily set as the variability range for all C.U. estimations, thus, no S.D. bars will be expressed with reported data.

Background values were determined by using the medium lysis value + 2 S.D. This method has been shown to give values very similar to those obtained with populations from syngeneic MLC's. Thus, a value of 4.25 ± 0.16 log C.U. was obtained from multiple syngeneic MLC's assayed on day 5 of culture, while the estimation based on medium lysis gave a value of 4.37 log C.U. .

2.3.3 Dose response and kinetic studies for the generation of cytotoxic T cells

The MLC experiments reported here, and most of those reported in subsequent chapters, were carried out with BALB/c spleen stimulator cells and CBA/H lymph node responder cells. Spleen cells were chosen as a source of stimulating cells for reasons of convenience, i.e., the spleen provided large numbers of lymphoid cells. Lymph nodes were used as a source of responder cells because they were generally found to give better responses than spleen cells (unpublished observations Davidson, Cooley, Lafferty). The kinetics of the cytotoxic response obtained with mixtures of these cells are shown in Figure 2.2. Thus, when the number of responders are held constant at 2×10^6/well the cytotoxic response peaked on days 5-6 for all concentrations of stimulators tested. Stimulation occurred over a four-fold concentration range of stimulator cells with 8×10^6 and 4×10^6 cells/well giving similar and slightly better responses than 2×10^6 cells/well. A wider range of stimulator cell concentrations was tested on day 5. From Figure 2.3 it can be seen that optimal stimulation only occurred over a narrow range of cell concentration values. The response dropped significantly when the stimulator concentration exceeded 8×10^6 cells/well or fell below 2×10^6 cells/well.

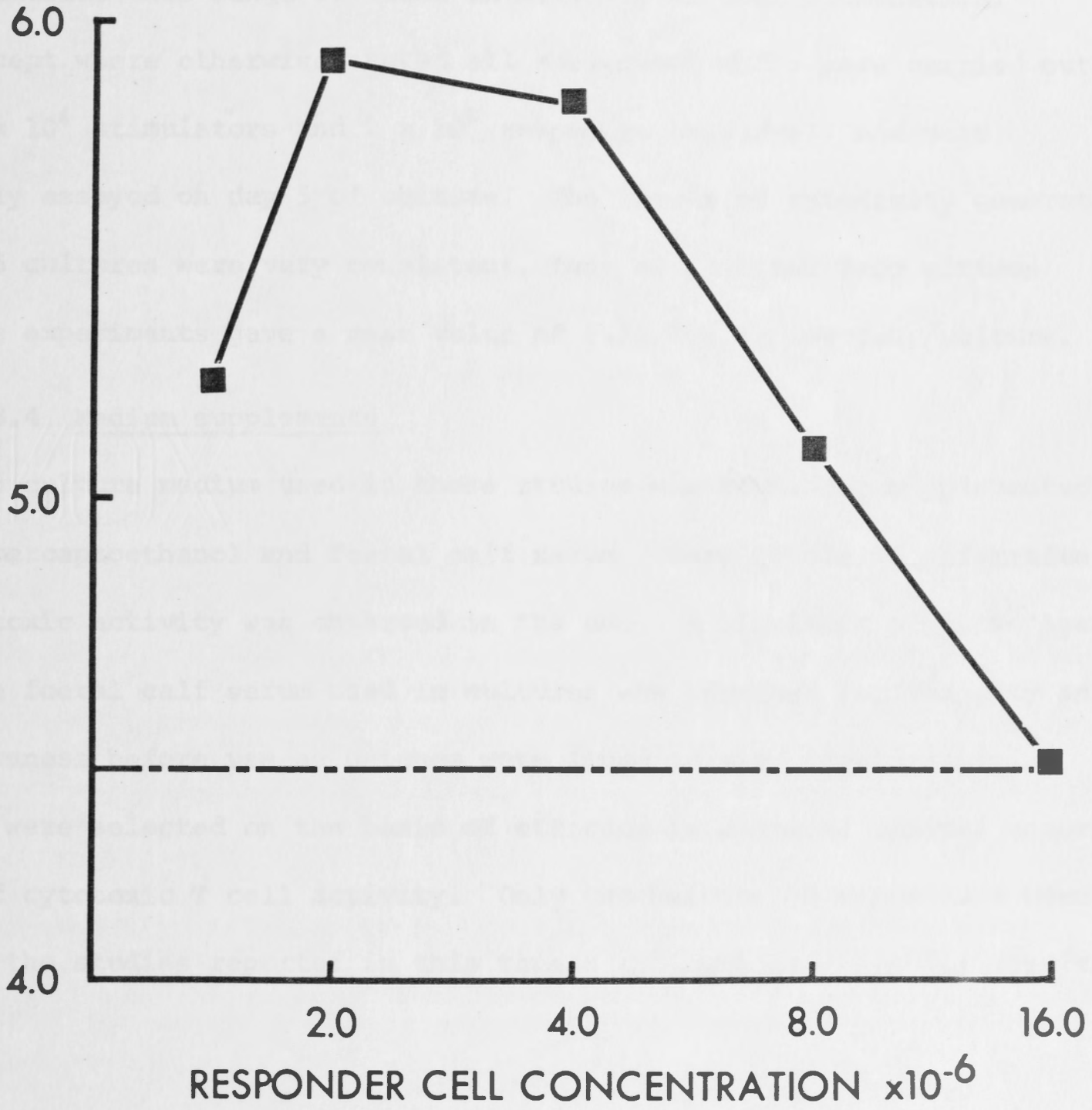

Figure 2.4 - Dose response curve obtained when 4 x 10^6 BALB/c spleen stimulator cells were cultured with different numbers of CBA/H lymph node responder cells. All cultures were assayed on day 5 of culture on ^{51}Cr labelled P815 targets. ▬ ▬ ▬ Background.

The optimal concentration of responder cells was determined on day 5. Figure 2.4 shows that, as with the stimulator population, an optimal response was only obtained over a narrow concentration range. Peak responses were observed between 2×10^6 and 4×10^6 lymph node cells/well. Values outside this range resulted in markedly reduced stimulation.

Except where otherwise stated all subsequent MLC's were carried out with 4×10^6 stimulators and 2×10^6 responder cells/well and were routinely assayed on day 5 of culture. The levels of cytoxicity generated in day 5 cultures were very consistent, thus estimations from sixteen separate experiments gave a mean value of 6.11 ± 0.12 log C.U./culture.

2.3.4 Medium supplements

The culture medium used in these studies was routinely supplemented with 2-mercaptoethanol and foetal calf serum. Very little proliferative or cytotoxic activity was observed in the absence of either of these agents.

The foetal calf serum used in cultures was screened for toxicity and effectiveness before use as batches were found to vary considerably. Batches were selected on the basis of efficacy in allowing maximal generation of cytotoxic T cell activity. Only two batches of serum have been used in the studies reported in this thesis and both gave similar results.

D I S C U S S I O N

The empirical observation of Henney (1971) that fractional specific lysis can be very accurately fitted with an expression of the form, $1 - e^{-KN}$ has allowed the derivation of a unit which is proportional to the number of killer cells in an effector population. Additionally, and inbuilt into the function is the ability to correct for loss of linearity for lysis values between 35 and 65%. The function derived here also bears similarity to that derived by Miller and Dunkley (1974).

The main advantage in expressing data in terms of cytotoxic units is that it allows valid comparisons to be made between different populations. Such comparisons cannot be made with data expressed in terms of chromium release because the latter is not linearly related to the number of cytotoxic cells in the effector population. Thus, direct comparison of percent ^{51}Cr release values may not reflect the different cytotoxic activities of individual cell populations. Expression of the data in terms of cytotoxic units per culture avoids tedious enumeration of effector populations and thereby, the biased concentration of weakly reactive populations. It is also particularly useful in the study of stimulator populations where the number of responder cells is held constant.

Since very high or very low values of specific lysis, which result in (1 - y) tending towards 0 or 1 respectively give very inaccurate estimations of cytotoxic activity, titrations of effector populations were routinely performed. Except where high or low values occurred this practice also ensured that multiple estimates of the one point were made.

The two main sources of variation in this adaptation of the MLC have been shown by Lafferty *et al.* (1974b) to be the foetal calf serum and the animals. The variation due to differences in batches of foetal calf serum was avoided by the use of a standard batch, while the animal variation was minimised by pooling the spleens of at least five animals

when preparing the stimulator population. Responder lymph node cells were also obtained by pooling the cells from five donors. It is also notable that only log phase P815 cells were used as targets in cytotoxicity assays as these were found to give much lower medium lysis values than cells taken later in the growth phase.

Stimulator populations were routinely incubated for 90' at 37°C prior to irradiation. This procedure has been shown by Lafferty *et al.* (1974b) to lead to considerably enhanced stimulation. The mechanism underlying this phenomenon is unknown.

In the past, *in vitro* assays of stimulation in mouse MLC have suffered from difficulties associated with the maintenance of cell viability (Häyry and Defendi, 1970). The report of Cheng and Hirsch (1972) that the addition of 2-mercaptoethanol (2-ME) to culture medium enhanced the survival of mouse lymphocytes suggested that this agent may be of benefit in the mouse MLC assay. The enhancement of both thymidine uptake (Heber-Katz and Click, 1972; Lafferty *et al.*, 1974b; Bevan *et al.*, 1974) and generation of cytotoxic T cells (Bevan *et al.*, 1974; Cerottini *et al.*, 1974; Lafferty *et al.*, 1974a) in MLC's following the addition of 2-ME has subsequently been reported. The mechanism of action of 2-ME is not clear.

Heber-Katz and Click (1972) reported that the sulph-hydryl group of 2-ME was the active moiety since three unrelated reducing agents produced similar enhancing effects. Engers *et al.* (1975a) on the other hand have shown that both oxidised and reduced 2-ME caused enhanced cytotoxic T cell responses, suggesting that the enhancing activity of 2-ME is not related to its reducing capacity. Similarly, there is also controversy over the kinetics of activity of 2-ME. Thus Heber-Katz and Click (1972) suggested that 2-ME affected early events in MLC since addition of the agent after 24 hours had little effect. In contrast, Engers *et al.* (1975a) have reported that addition of 2-ME can be delayed by up to 3 days after initiation of the culture without significant loss of cytotoxic activity.

These workers have suggested that one of the effects of 2-ME may be related to the differentiation of CTL precursor cells into active effector cells.

Whether 2-ME acts directly on lymphocytes or requires the presence of adherent cells is unknown. Bevan *et al.* (1974) have suggested that 2-ME may substitute for macrophages in the generation of cytotoxic T cells. Experiments described in later chapters indicate that in the culture system described here both accessory cells and 2-ME are required.

The degree of cytotoxic activity generated in MLC assays carried out in the presence of 2-ME was very much dependent on the cell density. Thus when the number of responding cells was held constant at 2×10^6/well and the density of the stimulator population was varied over a 16-fold range the cytotoxic activity passed through a maximum and then declined. The low levels of activity observed with less than 2×10^6 stimulator cells/well may be the result of "limiting antigen" or alternatively limiting numbers of accessory cells which are known to be required for the generation of cytotoxic T cells *in vitro* (Wagner *et al.*, 1972). The loss of activity observed with high numbers of stimulators may be due to the more obvious causes of medium depletion, accumulation of toxic products or overabundance of macrophages which are known to be toxic in culture (Rode and Gordon, 1974). Alternately, loss of activity may be attributed to tolerisation of the responding population or the selective generation of a suppressor population. The last two possibilities are probably remote, however, since all attempts to tolerise cytotoxic T cells in this laboratory have failed (data not shown) and although suppressor cells are known to be generated during responses to alloantigens both *in vivo* (Rich and Rich, 1974) and *in vitro* (Fitch *et al.*, 1976), there is no evidence that these cells are selectively induced in antigen excess. Similar dose response curves were observed when the number of stimulators was held constant and the number of responders varied. The first three explanations

suggested for the observations with the stimulator population also apply to the responder population. There is no evidence that the decline in the response observed with large numbers of responders is due to the presence of a suppressor population.

The kinetic studies described here coincide with those reported in other laboratories using similar culture conditions (Cerottini *et al.*, 1974; Fitch *et al.*, 1976) with peak cytotoxic activity occurring between day 5 and 6 of culture. The rapid fall in activity after day 6 has been attributed to the conversion of cytotoxic T cells to memory cells (MacDonald *et al.*, 1974a,b) and possibly to the presence of a regulator or suppressor population (Fitch *et al.*, 1976).

The dose response and kinetic studies described here indicate the importance of characterising each component of the MLC. Thus preliminary dose response and kinetic studies have been performed whenever investigations with a new stimulator or responder population have been initiated.

S U M M A R Y

A method for quantitating cytotoxic T cell activity *in vitro* has been described. The standardisation of both the MLC procedure and the cytotoxicity assay has led to minimisation of sources of variation and thus made the procedures suitable for quantitative studies. Except where otherwise stated a standard MLC procedure has been adopted using 4×10^6 spleen stimulators and 2×10^6 lymph node responders per well, and assaying cytotoxic activity on day 5 of culture.

Cytotoxic activity was routinely expressed in terms of log cytotoxic units per culture. The standard deviation (S.D.) of replicate cultures was found to be 0.06 log cytotoxic units. Any values differing by more than two standard deviations, i.e. 0.12 log cytotoxic units, were considered to be significantly different. This policy was adopted for the data presented in all subsequent chapters.

CHAPTER 3

THE CAPACITY OF VARIOUS CELL POPULATIONS TO INDUCE CYTOTOXIC T CELL RESPONSES *IN VITRO*

3.1 Introduction

For the induction of optimal primary proliferative and cytotoxic T cell responses *in vitro*, alloantigens must be presented on the surface of stimulator cells (Wagner and Boyle, 1972; Engers *et al.*, 1975b Lafferty *et al.*, 1974a; Häyry and Andersson, 1976; Wagner and Röllinghoff, 1976). Although some exceptions exist it is generally agreed that lymphoid populations and some neoplastic cell lines are a better source of stimulator cells than non-lymphoid cells (Wagner and Wyss, 1973; Rode and Gordon, 1974; Lafferty *et al.*, 1975; Wagner and Röllinghoff, 1975). However, whether all subpopulations of lymphoid cells, T cells, B cells and cells of the macrophage/monocyte series have an inherent capacity to stimulate remains controversial.

While there is general agreement that Ig^+ cells are able to induce both proliferative (Cheers and Sprent, 1973; Plate and McKenzie, 1973; von Boehmer, 1974; Lonai and McDevitt, 1974; Lohrman *et al.*, 1974; Simpson, 1975; Sondel *et al.*, 1975) and cytotoxic T cell (Wagner and Wyss, 1973; Simpson, 1975; Sondel *et al.*, 1975) responses in mice and humans there is still debate over the stimulator potential of Ig^- cells. Accordingly, some investigators have observed significant proliferative (Cheers and Sprent, 1973; von Boehmer, 1974; Lonai and McDevitt, 1974; Sondel *et al.*, 1975) and cytotoxic T cell (Wagner and Wyss, 1973; Sondel *et al.*, 1975) responses to Ig^- cells while others have been unable to demonstrate such activity (Plate and McKenzie, 1973; Simpson, 1975; Lohrman *et al.*, 1974). The apparent contradictions found in these studies may in some way relate to differences in MLC methodology or cell purification techniques.

Conflicting results have also been obtained with mouse macrophages as a source of stimulators, since it has been shown that macrophages stimulate better than lymphocytes (Talmage and Hemmingson, 1975; Schirrmacher *et al.*,

1975), that macrophages and lymphocytes stimulate equally (Simpson, 1975) and that lymphocytes stimulate better than macrophages (Wagner and Wyss, 1973).

The following studies, using the standardised MLC procedures described in the previous chapter and a variety of cell separation techniques were undertaken to determine the comparative ability of lymphoid populations from various sources, neoplastic cell lines, Ig^- cells, Ig^+ cells and macrophages to induce cytotoxic T cell responses. Experiments were also carried out to determine whether the presence of Fc receptors, complement receptors or Ia antigens conferred superior stimulator potential on lymphocytes.

3.2 Methods and materials

3.2.1 Animals

8-10 week old CBA/H, BALB/c, B6.H-2^b, B6.C-H-2^{ba} A.TH and A.TL mice were obtained from the breeding colonies of this school.

3.2.2 Tissue culture procedures

Mixed lymphocyte cultures and cytotoxicity assays were performed as described in Chapter 2. Macrophage targets were prepared by culturing peritoneal macrophages in Eagle's Minimal Essential Medium (Cat. No.F-15, GIBCO, Grand Island, NY, USA) containing antibiotics and 10% heat inactivated foetal calf serum at a concentration of 1×10^6/well in 6 mm diameter culture trays (Linbro model IS-FB-96-TC Multidish Disposo Trays) overnight at 37°C in 10% CO_2, 7% O_2 and 83% N_2. Monolayers were washed thoroughly and labelled in the wells by adding 7.5 µCi ^{51}Cr/well in 50 µl of F-15 medium. Plates were incubated for 1 hour at 37°C and then washed thoroughly with warm complete F-15 medium. Killer cell populations were added to target cells in a total volume of 0.2 ml. The cultures were incubated for 4 hours or overnight at 37°C in 10% CO_2, 7% O_2 and 83% N_2. All assays were set up in quadruplicate and 0.1 ml of the supernatant was counted. Water

lysis and medium lysis values were determined as described for the labelling of P815 cells (see section 2.2.5).

L-929 cells were labelled in suspension at a concentration of 5×10^6 cells in 0.4 ml of F-15 containing 300 µCi ^{51}Cr. Cells were incubated for 1 hour at 37°C with frequent mixing and then thoroughly washed. Labelled cells were dispensed into flat bottomed 6 mm Linbro culture trays at a concentration of 2×10^4 cells/well. Immune cells were added to target cells in a total volume of 0.2 ml and following a 4 hour incubation period in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2, 0.1 ml of the supernatant was counted. Water and medium lysis values were estimated as described for P815 cells (see section 2.2.5).

3.2.3 Neoplastic cell lines

Continuous line mouse fibroblasts (L-929) (Sanford *et al.*, 1948) were grown in MEM with 5% FCS and 100 µg/ml of penicillin and streptomycin and incubated at 37°C in a humidified atmosphere. IgG and IgA plasmacytoma cells obtained from BALB/c mice were the gift of Dr Noel Warner. These cells were maintained in MEM with 5% FCS and 100 µg/ml of penicillin and streptomycin and were incubated in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. Cultures were fed every 48 hours.

3.2.4 Carbonyl iron treatment

Cell suspensions at 2×10^7/ml were mixed with enough carbonyl iron powder ($Fe_3(CO)_{12}$), Atomergic Chemetals Co., New York, to render the solution dark grey. The mixtures were then rotated (30 revs/min) for 1 hour at 37°C. The iron adherent cells were removed by an electro-magnet. The iron powder was washed twice to recover non-specifically trapped cells. Recoveries varied according to the tissue treated and viabilities were >95%.

3.2.5 Preparation of anti-θ reagent

Anti-θ ascitic fluid was prepared in AKR/J mice which were given up to 10 intraperitoneal injections of 5 - 10 x 10^7 CBA thymocytes at weekly intervals. The first injection was given with 10^9 *B. pertussis* organisms. Three days before the final dose of thymocytes, the mice were injected intraperitoneally with 0.2 ml of a 10% suspension of sarcoma 180 cells in saline (Tikasingh *et al.*, 1966). The ascitic fluid was harvested 7 days after the final thymocyte injection. Unimmunised AKR/J mice were used as donors of control ascitic fluid. The anti-θ ascitic fluid was cytotoxic for >98% of thymus cells and for 20% to 30% of spleen cells.

3.2.6 Treatment of cells with anti-θ and complement

Cells were incubated (37°C, 30 min.) at a concentration of 5 x 10^7 - 1 x 10^8 cells/ml with a 1:4 dilution of anti-θ ascitic fluid, washed and resuspended in a 1:3 dilution of guinea pig serum, previously absorbed on agarose (80 mg/ml, 1 hour, 0°C). Cells were then incubated for a further 15 minutes at 37°C, washed and recounted. Viability was judged by trypan blue (0.05%) dye exclusion.

3.2.7 Treatment of cells with anti-Ia serum and complement

Cells bearing Ia antigens were eliminated by treatment with A.TH anti-A.TL serum and rabbit complement as described by McKenzie and Parish (1976). Briefly, 1.5 x 10^8 cells in 1 ml of medium (F-15 + 10% FCS) were incubated with 1.0 ml of anti-Ia 1, 2, 3, 7 at 1:2 dilution for 30' at 37°C. 10 ml of medium were added and the cells sedimented. 1.3 ml of rabbit complement (RC), (1:3 dilution) was added and a further 30' period of incubation at 37°C performed. Cells were then washed twice in medium and their viability assessed by trypan blue exclusion. After treatment with anti-Ia serum and complement 53% of CBA/H spleen cells were lysed while RC treatment alone lysed only 8%.

3.2.8 Preparation of cell suspensions

Spleen and lymph node populations were prepared as described in Chapter 2. Mouse peritoneal cells (PC) were obtained by injecting 4 ml of cold PBS IP. The abdomen was then gently massaged and the inoculum aspirated. The cells were centrifuged in siliconised glass tubes at 4°C for 5' at 400 g then resuspended in cold culture medium. Viable cells were counted by trypan blue exclusion. Adherent cell monolayers were prepared by incubating PC at various concentrations in 24 well Linbro trays for 4 hours at 37°C. Non-adherent cells were removed by vigorous washing with warm (37°C) culture medium. Between 50% and 60% of PC were found to be adherent by this method. Adherent spleen cell monolayers were prepared in a similar way. In this case 20% to 30% of the population was adherent. Non-adherent cells were collected from centrifuged washings.

Bone marrow cells were flushed from femurs and tibias with a syringe and dispersed by repeated aspiration. They were then washed, declumped and counted as for spleen and LN cells (Chapter 2). Peripheral blood leukocytes (PBL's) were prepared by collecting blood in Alsevers solution. The erythrocytes were removed by layering the cell suspension on to the separating medium (Isopaque/Ficoll) described in section 3.2.9.3. Following centrifugation the purified PBL's were washed three times and counted.

3.2.9 Cell separation procedures

Cells bearing surface immunoglobulin (Ig^+), theta antigen (θ^+) or complement (CR^+) or Fc (FcR^+) receptors were removed from lymphoid cell suspensions by forming rosettes and separating the rosetting and non-rosetting cells by centrifugation in Isopaque/Ficoll. The methods used have been described elsewhere (Parish and Hayward, 1974; Parish *et al.*, 1974; Parish and Chilcott, 1975) and are also detailed below.

3.2.9.1 Antisera - Antisera to mouse IgG and rabbit IgG were prepared by Dr C.R. Parish. Briefly, mouse IgG and rabbit IgG were obtained from $(NH_4)_2\ SO_4$ precipitation (40% saturation) of mouse or rabbit serum respectively. After extensive washing the precipitate was dissolved in a 20 mM sodium phosphate buffer, pH 8.0 and passed through a DEAE column eluted with the same buffer. The protein eluted was then concentrated by pressure dialysis, applied to a sephadex G-200 column and the IgG fraction from the column collected and concentrated.

Antisera to rabbit Ig were raised in outbred merino sheep by injecting, into multiple sites, 1 mg of rabbit IgG emulsified in FCA (1.0 ml/sheep of a 1:1 mixture of FCA and antigen). Four to six weeks later the animals were boosted with 1 mg of rabbit IgG in FIA and the sheep were bled out 7 - 14 days after the secondary challenge. The globulin fraction of this antiserum was precipitated with $(NH_4)_2\ SO_4$ at 40% saturation and after extensive washing the precipitate was redissolved in 0.15 M NaCl.

Antisera to mouse Ig were raised in rabbits by an identical immunisation schedule.

3.2.9.2 Rosetting procedures -

A. Surface immunoglobulin - The procedure for rosetting mouse lymphocytes for surface immunoglobulin consisted of two stages: first, reaction of the lymphocytes with rabbit anti-mouse Ig and second, rosetting of the lymphocytes with SRBC coated with sheep IgG specific for rabbit Ig.

To 2.5 ml of spleen cells at 4×10^7 cells/ml in complete medium was added 10 µl of hyperimmune rabbit anti-mouse Ig serum. This antiserum had been titrated against spleen cells and a dilution selected at which the number of Ig rosetting cells had reached a plateau. The cell antiserum mixture was incubated on ice for 30 minutes and then centrifuged at 300 g for 5' at 4°. The cell pellet was resuspended in 2.5 ml of medium and 2.5 ml of a 20% suspension of SRBC (which had been previously coated using $Cr\ Cl_3$, with sheep IgG specific for rabbit Ig) was added. The method of

coating consisted of adding 10 µl of the sheep anti-rabbit Ig to 0.25 ml of packed SRBC in 4 ml of saline; the red cell suspension was mixed and immediately 0.25 ml of Cr Cl_3 was added with constant shaking. The mixture was left to react at room temperature for 10 minutes and then unreacted Cr Cl_3 inactivated by adding two volumes of PBS. The coupled red cells were washed twice in PBS and resuspended to make a 20% suspension.

The lymphocyte-erythrocyte mixture was then centrifuged at 300g for 5 minutes at 4°C to enable rosette formation. Sodium azide (20 µl at 25%) was added just before resuspending the cell pellet. Samples were taken and diluted in PBS containing 1% (V/V) glutaraldehyde and 2% (W/V) crystal violet. Any lymphocyte which bound >5 erythrocytes was counted as a rosette. By the above method 45% - 52% of spleen cells were Ig^+.

B. Fc rosetting - Equal volumes of a 5% suspension of washed SRBC in PBS and an optimal dilution of mouse anti-SRBC antibody in PBS were incubated at 37°C for 30 minutes. The sensitised cells were washed three times with PBS. A 20% suspension of sensitised red cells in medium was prepared and stored on ice. Rosettes were formed by mixing 2.5 ml of cells at a concentration of 4×10^7/ml with 2.5 ml of a 20% suspension of sensitised red cells. The mixture was incubated at 37°C for 10 minutes then spun at 20°C for 5 minutes at 300 g. Sodium azide (20 µl of 25%) was added just before resuspending the cell pellet. Samples were taken and counted as for Ig rosettes. 40% - 50% of spleen cells formed Fc rosettes.

C. Complement rosetting - Equal volumes of a 5% SRBC suspension in PBS and an optimal dilution (previously determined) of rat anti-SRBC antibody in PBS were incubated at 37°C for 30 minutes. The sensitised cells were washed twice with PBS and resuspended to 5% concentration. Equal volumes of 5% antibody coated SRBC and a 1/10 dilution of fresh mouse serum, which acted as a source of complement, were then incubated at 37°C for 20 minutes. The sensitised red cells were then washed twice with PBS and resuspended in culture medium to a final concentration of 20%.

Rosetting was performed as for Fc rosettes (section 3.2.9.2.B). 20% - 30% of spleen cells were found to be CR^+.

D. Ig and theta (θ) rosetting - 0.5 ml of anti-θ serum was added to 2.5 ml of spleen cells at a concentration of 4×10^7/ml. The cells were incubated on ice for 30 minutes, washed twice and resuspended in 2.5 ml medium. The cells were then mixed with 10 µl of rabbit anti-mouse Ig antiserum, incubated on ice for 30 minutes, washed once and resuspended in 2.5 ml of complete medium. The cells were then rosetted as for surface immunoglobulin (3.2.9.2.A). 70% - 75% of spleen cells were found to be $Ig^+\theta^+$ by this method. The $Ig^-\theta^-$ cells consist of K cells, null cells and macrophages.

3.2.9.3 Separation of rosetting and non-rosetting cells

The method of separation was based on a procedure described by Böyum (1968), for obtaining lymphocytes from peripheral blood.

The rosetting of cells with different surface markers was carried out as described in the preceding sections. 5 ml of each preparation was brought to 20°C and layered gently on to 4 ml of separating medium prewarmed to 20°C which consisted of 12 parts of 14% (mass/vol) Ficoll (Pharmacia, Uppsala, Sweden) dissolved in distilled water and 5 parts of 32.8% (mass/vol) sodium metrizoate (Isopaque; Nyegaard and Co., Oslo, Norway). This mixture had a density of 1.09 and is referred to as Isopaque/Ficoll. The separations were carried out in U-bottomed siliconised polycarbonate centrifuge tubes, 15 mm in diameter. The tubes were centrifuged at 20°C for 20 minutes at 1200 g. After centrifugation the supernatant above the Isopaque/Ficoll interface was discarded and the lymphocyte layer together with all the separating medium above the red cell pellet was collected. The lymphocyte fraction was diluted to 10 ml with complete medium, mixed well, and the lymphocytes pelleted by centrifugation at 300 g for 10 minutes. The cells were resuspended in 5 ml of complete medium and counted.

3.2.10 Preparation of neoplastic stimulator cells

P815-X2 mastocytoma cells and IgA and IgG plasmacytoma cells were irradiated (5000 R) prior to use as stimulator cells. L-929 cells were treated with mitomycin C as follows, 20 µg/ml of mitomycin C (Calbiochem, Los Angeles, California) was added to L cells at 10^7/ml and the mixture incubated at 37°C for 30 minutes. After two washes the cells were suspended in culture medium to the desired concentration.

3.2.11 Estimation of thymidine uptake by cultured cells

Mixed lymphocyte cultures set up in 24 well Linbro trays were mixed well after 4 or 5 days incubation and 0.2 ml aliquots removed from each well and dispensed into flat bottomed 6 mm Linbro culture trays. 25 µl of [^{3}H] thymidine (Amersham, TRA 120, 5 ci/mM at a concentration of 150 µCi/ml in PBS was added to each culture. After a further incubation period of 5 hrs at 37°C in a humidified atmosphere of 10% CO_2, 7% O_2, 83% N_2 the cultures were harvested on a MASH II harvester (Microbiological Associates) onto glass fibre filter discs. These discs were then dried in an oven at 100°C for 20 minutes. Each disc was then placed in a vial containing 8.0 ml of scintillation fluid (0.5% PPO, 2,4 diphenyloxazole in toluene) and its content of tritium estimated in a Packard liquid scintillation counter.

3.3 Results

3.3.1 The tissue distribution of stimulator cells

Except where otherwise stated all lymphoid stimulator cells were obtained from BALB/c (H-2^d) mice and CBA/H (H-2^k) lymphoid cells, at a concentration of 2 x 10^6/culture, were used as responders. Dose response and kinetic studies were routinely performed.

A. Lymphoid and neoplastic cells - The results of a comparative study of the stimulator capacity of various lymphoid populations and neoplastic cell lines are summarised in Table 3.1.

TABLE 3.1

THE STIMULATOR CAPACITY OF VARIOUS LYMPHOID AND NEOPLASTIC CELL POPULATIONS

Stimulator cell population	Optimal stimulator concentration/culture	Cytotoxic activity log C.U./culture	Background log C.U./culture
Spleen[a]	4×10^6	6.08	4.20
Lymph node	4×10^6	6.00	"
Peripheral blood lymphocytes	2×10^6	5.85	"
Bone marrow	4×10^6	5.99	"
Thymus	4×10^6	5.33	"
Peritoneal cells[a]	2×10^6	4.56	4.11
	1×10^6	4.33	"
	5×10^5	5.13	"
	2×10^5	4.72	"
P815 mastocytoma cells[b]	5×10^5	Bg[d]	4.18
	2×10^5	4.58	
	1×10^5	5.62	
	5×10^4	5.20	
L cells[c]	1×10^5	5.61	4.37
	5×10^4	4.79	
	1×10^4	4.46	
IgA plasmacytoma cells[b]	1×10^6	5.09	4.18
	5×10^5	5.51	
	1×10^5	5.06	
IgG plasmacytoma cells[b]	1×10^6- 5×10^4	Bg	4.18

a Irradiated BALB/c stimulator cells cultured with 2×10^6 CBA/H lymph node responder cells and assayed on day 5 on ^{51}Cr labelled P815 targets.

b Irradiated (5000 R) P815-X2 mastocytoma cells or IgA or IgG ($\underline{H\text{-}2}^d$) plasmacytoma cells were cultured for 5 days with 2×10^6 CBA/H lymph node cells then assayed on ^{51}Cr labelled P815 cells.

c Mitomycin C treated L-929 cells cultured with 2×10^6 BALB/c lymph node cells for 5 days then assayed on ^{51}Cr labelled L cells in a 4 hr assay.

d Bg = Background.

Since the dose response data with all lymphoid stimulator cell populations except peritoneal cells parallels that already described for spleen cells (Chapter 2, Figure 2.3), only values obtained with optimal concentrations of these stimulator cells have been tabulated.

With the exception of an IgG plasmacytoma cell line, all cells tested, both lymphoid and neoplastic, were capable of stimulating significant cytotoxic T cell responses. Spleen, bone marrow, peripheral blood and lymph node cells induced about twice as much cytotoxic activity (double the number of cytotoxic units/culture) as P815 mastocytoma cells, L cells and IgA plasmacytoma cells and 5-8 times more than thymus and peritoneal cells. Peak cytotoxic activity was observed on day 5 with all stimulator populations except thymus cells, where the peak occurred a day later.

It is notable that maximal stimulation with neoplastic and peritoneal cells occurred with 10-40 times fewer cells than were required for maximal stimulation with lymphoid cells. The dose response curves also proved to be different. Whereas stimulation with lymphoid cells (excluding peritoneal cells) occurred over at least an 8-fold concentration range (see Figure 2.3, Chapter 2), stimulation with neoplastic and peritoneal cells was only observed after a narrow concentration range. Accordingly, cytotoxic activity often dropped dramatically when the stimulator cell concentration giving optimal responses was increased or decreased as little as 2-fold (Table 3.1).

B. Adherent and non-adherent lymphoid cells - Adherent, non-adherent and untreated spleen and peritoneal cells were tested for their stimulator activity. In these studies adherent cells were those cells which remained attached to plastic tissue culture wells after a 4 hour incubation period followed by thorough washing.

As the data in Table 3.2 indicates all of the spleen cell populations, plastic adherent and non-adherent, and untreated induced similarly high levels of cytotoxic activity. In comparison, untreated and plastic adherent

TABLE 3.2

THE ABILITY OF PLASTIC ADHERENT AND NON-ADHERENT BALB/c SPLEEN AND PERITONEAL CELLS TO STIMULATE

Stimulator population	Stimulator[c] concentration/ well	Cytotoxic activity[a] log C.U./culture	Background log C.U./ culture
Peritoneal cells	2×10^5	5.13	4.66
Plastic adherent peritoneal cells	2×10^5	5.23	"
Non-plastic[b] adherent peritoneal cells	1×10^6	5.57	"
Spleen	4×10^6	6.16	4.37
Plastic adherent spleen cells	$\sim 1 \times 10^6$	6.17	"
Non-plastic[b] adherent spleen cells	$\sim 3 \times 10^6$	6.15	"

a 2×10^6 CBA/H lymph node cells were used as responders throughout. 2 S.D. for all estimations was 0.12 log C.U.

b Non-adherent cells were those cells which did not adhere to plastic during 4 hours incubation at 37°C.

c Cell numbers giving maximal stimulation of cytotoxic activity.

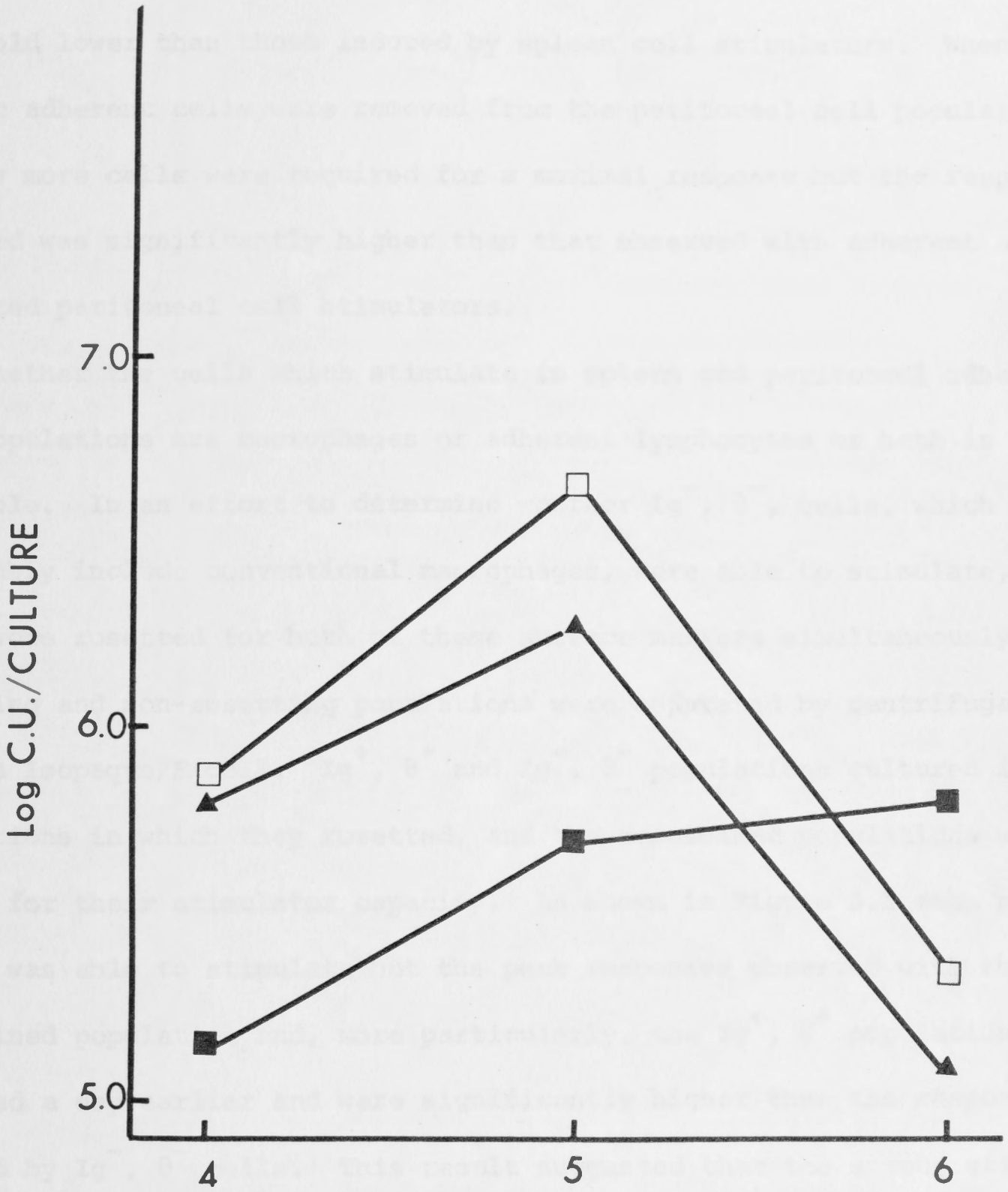

Figure 3.1 - The stimulator activity of Ig^+, θ^+ and Ig^-, θ^- BALB/c spleen subpopulations.

▲—▲ , 4 x 10^6 Ig^+, θ^+ + Ig^-, θ^- (recombined population) cells

■—■ , 1 x 10^6 Ig^-, θ^- cells

□—□ , 3 x 10^6 Ig^+, θ^+ cells

All stimulator cells were irradiated and cultured with 2 x 10^6 CBA/H lymph node cells. Background = 4.8 log C.U. % Ig^+, θ^+ cells = 75%.

peritoneal cells stimulated responses significantly above background but 8-10 fold lower than those induced by spleen cell stimulators. When plastic adherent cells were removed from the peritoneal cell population 5 times more cells were required for a maximal response but the response obtained was significantly higher than that observed with adherent and untreated peritoneal cell stimulators.

Whether the cells which stimulate in spleen and peritoneal adherent cell populations are macrophages or adherent lymphocytes or both is debatable. In an effort to determine whether Ig^-, θ^-, cells, which presumably include conventional macrophages, were able to stimulate, spleen cells were rosetted for both of these surface markers simultaneously. The rosetting and non-rosetting populations were separated by centrifugation through Isopaque/Ficoll. Ig^+, θ^+ and Ig^-, θ^- populations cultured in the proportions in which they rosetted, and the recombined populations were all tested for their stimulator capacity. As shown in Figure 3.1 each population was able to stimulate but the peak responses observed with the recombined population and, more particularly, the Ig^+, θ^+ population occurred a day earlier and were significantly higher than the response induced by Ig^-, θ^- cells. This result suggested that the strong stimulation observed with plastic adherent spleen cells (Table 3.2) may not be completely attributable to conventional macrophages or other Ig^-, θ^- cells. The apparent enhancement of stimulator activity following the removal of Ig^-, θ^- cells from the stimulator population was not a consistent finding.

Purification of macrophages often rests on two properties generally considered characteristic of this population, namely, radiation resistance and prolonged viability in culture. When plastic-adherent peritoneal cell populations were cultured under conventional conditions for as little as 24 hours and then thoroughly washed their capacity to stimulate was observed to decrease dramatically (Table 3.3). A similar loss in stimulator activity was observed with irradiated or untreated Ig^-, θ^- peritoneal

TABLE 3.3

LOSS OF STIMULATOR ACTIVITY BY BALB/c PERITONEAL CELLS FOLLOWING CULTURE

Culture interval[a]	Peritoneal cell concentration/well	Cytotoxic activity[b] log C.U./ culture
4 hrs	5×10^4	4.72
	2×10^5	5.23
	5×10^5	4.23
24 hrs	5×10^4	Bg
	2×10^5	"
	5×10^5	"
72 hrs	5×10^4	Bg
	2×10^5	"
	5×10^5	"

a Culture interval prior to use as stimulator cells.

b Cytotoxic activity estimated by ^{51}Cr release assay using 1×10^5 labelled P815 cells. 2 S.D. = 0.12 log C.U.

c Background value (Bg) = 4.66 log C.U.

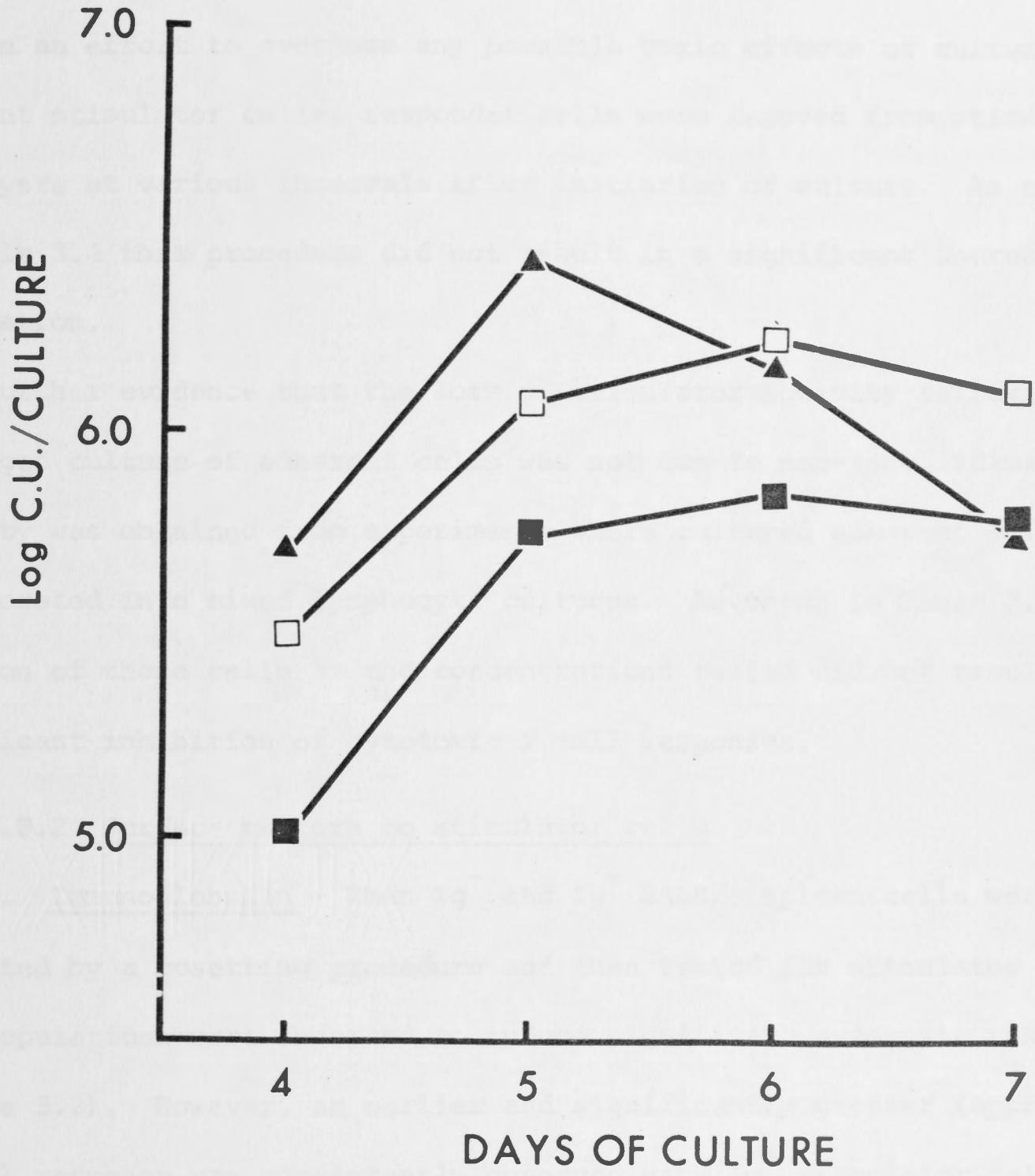

Figure 3.2 - The stimulator activity of Ig^+ and Ig^- BALB/c spleen sub-populations.

4×10^6 irradiated BALB/c stimulator cells were routinely cultured with 2×10^6 CBA/H lymph node responder cells.

▲—▲ , Ig^+ cells

□—□ , Unfractionated cells

■—■ , Ig^- cells

Background = 4.20 log C.U. % Ig^+ cells = 48%.

cells cultured for three days prior to the addition of responder cells. In both cases the cultured adherent cells were viable, able to phagocytose and morphologically resembled macrophages.

In an effort to overcome any possible toxic effects of cultured adherent stimulator cells, responder cells were removed from stimulator monolayers at various intervals after initiation of culture. As evidenced in Table 3.4 this procedure did not result in a significant increase in stimulation.

Further evidence that the loss of stimulator activity following prolonged culture of adherent cells was not due to non-specific macrophage toxocity was obtained from experiments where cultured adherent cells were incorporated into mixed lymphocyte cultures. As shown in Table 3.4 the addition of these cells at the concentrations tested did not result in significant inhibition of cytotoxic T cell responses.

3.3.2 Surface markers on stimulator cells

A. Immunoglobulin - When Ig^- and Ig^+ BALB/c spleen cells were separated by a rosetting procedure and then tested for stimulator potential both populations were observed to induce significant cytotoxic responses (Figure 3.2). However, an earlier and significantly greater (approximately 4-fold) response was consistently observed with Ig^+ stimulator cells. Ig^+ cells were also slightly better stimulators than unfractionated spleen cells.

Interestingly, when Ig^+ and Ig^- cells were treated with carbonyl iron powder prior to culture to remove adherent cells, and the adherent population was replaced with responder adherent cells, both populations induced similar levels of cytotoxic activity which in both instances peaked on day 5 of culture (Table 3.5).

TABLE 3.4

TOXICITY OF CULTURED PLASTIC-ADHERENT BALB/c PERITONEAL CELLS

Peritoneal cell concentration/ well[a]	Contact interval	Log C.U./culture	Background log C.U./ culture
A. 2×10^5 1×10^5 5×10^4 1×10^4	4 hrs[b]	Bg[d]	4.20
"	12 hrs	Bg	"
"	24 hrs	Bg	"
"	48 hrs	Bg	"
B. 5×10^5	5 days[c]	6.30	4.84
1×10^5	"	6.35	"
5×10^4	"	6.42	"
1×10^4	"	6.40	"
None	"	6.38	"

a Approximately 60% of peritoneal cells adhere to plastic. BALB/c adherent cells cultured for 3 days prior to use.

b 2×10^6 CBA/H lymph node cells were cultured with BALB/c macrophages for the intervals specified. All cultures were assayed on day 5.

c 4×10^6 irradiated BALB/c spleen stimulators and 2×10^6 CBA/H lymph node responders were cultured on BALB/c macrophage monolayers for 5 days.

d Bg = Background.

TABLE 3.5

ACCESSORY CELL REQUIREMENTS FOR THE RESPONSE TO Ig^+ AND Ig^- SPLENIC STIMULATOR POPULATIONS

Stimulator population[a]	Carbonyl iron treatment of stimulator population	Addition of CBA/H spleen accessory cells	Cytotoxic activity log C.U./ culture
Ig^+ [d]	-[b]	-[c]	6.16[e]
Ig^-	-	-	5.61
Ig^+	+	-	4.69
Ig^-	+	-	4.69
Ig^+	+	+	5.82
Ig^-	+	+	6.05

a 2×10^6 irradiated BALB/c Ig^+ or Ig^- stimulator cells and 2×10^6 CBA/H lymph node responder cells were used throughout.

b - = not treated; + = treated.

c - = no accessory cells added; + = 2×10^6 irradiated CBA/H spleen cells added.

d Rosetting % = 45%;

e Background = 4.20 log C.U.

B. Fc and complement receptors - Separation of lymphocytes into subpopulations on the basis of Fc receptors, like separation on the basis of immunoglobulin, did not differentiate stimulator from non-stimulator populations. Thus Fc^+ and Fc^- subpopulations both induced similar levels of cytotoxicity (Table 3.6). Similarly, Ig^+ cells with and without Fc receptors and Ig^- cells without Fc receptors all stimulated responses which did not differ significantly from each other or from unfractionated controls (Table 3.5). Preliminary experiments with complement receptor bearing (CR^+) cells have indicated that this surface marker like Fc receptors and surface immunoglobulin is not unique to stimulator cells. However, in contrast to studies with the latter markers neither CR^+ nor CR^- cells stimulated as well as unfractionated cells (Table 3.6).

C. Ia antigens - The effects of the pre-treatment of CBA/H stimulator spleen cells with A.TH anti A.TL serum (anti-Ia serum) and complement prior to culture with BALB/c lymph node responder cells are summarised in Table 3.7. Although 47% of the cells were lysed by this treatment the residual population stimulated as well as the untreated population.

Evidence that an I region difference is not required for the generation of cytotoxic T cells is summarised in Table 3.8. In this study significant proliferative and cytotoxic responses by A.TL lymph node cells were observed following stimulation with CBA/H spleen cells. These populations are non-congenic but share the I region. The further addition of irradiated A.TH spleen cells which differ from A.TL cells only at the I region enhanced both the proliferative and cytotoxic responses significantly (Table 3.8). This enhancement was not observed when irradiated A.TL spleen cells were added.

Furthermore, significant specific cytotoxic T cell responses were also observed when mutant B6.C-H-2^{ba} spleen cells were used to stimulate B6-H-2^{b} lymph node responder cells which differ from the former by a point mutation at the K end of the H-2 complex (Table 3.8).

TABLE 3.6

SURFACE MARKERS ON STIMULATOR CELLS

Stimulator population[a]	Cytotoxic activity[b] log C.U./culture	Background log C.U./culture
FcR^{+} [c]	5.88	3.84
FcR^{-}	6.20	"
Ig^{+} FcR^{+}	5.86	"
Ig^{+} FcR^{-}	6.17	"
Ig^{-} FcR^{-}	5.82	"
Unfractionated	6.09	"
CR^{+} [d]	5.14	4.26
CR^{-}	5.28	"
Unfractionated	5.60	"

a 4×10^6 irradiated BALB/c stimulator cells used routinely with 2×10^6 CBA/H lymph node responder cells.

b All cultures assayed day 5 on ^{51}Cr labelled P815 targets.

c FcR rosetting % = 40%.

d CR rosetting % = 23%.

TABLE 3.7

ANTI-Ia SERUM AND COMPLEMENT TREATMENT

OF CBA/H STIMULATOR POPULATION

Treatment of stimulator population	% viability following treatment[c]	Cytotoxic activity[d] log C.U./culture
Anti Ia serum + complement[b]	53%	5.00
Complement only	92%	5.11
Untreated	98%	5.07

a 4×10^6 CBA/H spleen cells used as stimulators and 2×10^6 BALB/c lymph node cells as responders.

b A.TH anti-A.TL serum + rabbit complement.

c Viability estimated by trypan blue exclusion.

d 2×10^4 ^{51}Cr labelled L cells used as targets.
Background activity = 3.64 log C.U.

TABLE 3.8

GENERATION OF CYTOTOXIC T CELLS IN THE PRESENCE AND ABSENCE OF AN H-2I REGION DIFFERENCE

	Stimulator spleen cell[a] combinations	Cytotoxic activity log C.U./culture[c]	^{3}H-T uptake[b] c.p.m.
A.	4×10^6 CBA/H	5.93	30, 183
	4×10^6 CBA/H + 4×10^6 A.TH	6.42	40, 728
	4×10^6 CBA/H + 2×10^6 A.TH	6.48	72, 849
	4×10^6 CBA/H + 1×10^6 A.TH	6.65	76, 873
	4×10^6 CBA/H + 4×10^6 A.TL	6.08	40, 169
	4×10^6 CBA/H + 2×10^6 A.TL	6.19	42, 059
	4×10^6 CBA/H + 1×10^6 A.TL	6.11	33, 556
	4×10^6 A.TH	5.25	55, 783
	2×10^6 A.TH	5.00	39, 670
	1×10^6 A.TH	4.26	23, 785
	4×10^6 A.TL	Bg	3, 920
	2×10^6 A.TL	Bg	1, 576
	1×10^6 A.TL	Bg	1, 542
B.	B6.C-H-2^{ba} [f]	5.70[d]	N.T.
	B6.C-H-2^{ba}	4.80[e]	N.T.

a 2×10^6 A.TL (s kkkk d) lymph node responder cells were used routinely CBA/H (k kkkk k) and A.TH (s ssss d) stimulator cells were both irradiated (1000 R).

b Tritiated thymidine (^{3}H-T) uptake measured day 5 of culture S.E. = ±5%.

c Cultures assayed on 2×10^4 ^{51}Cr labelled L cells, incubation period, 4 hrs. Background = 4.20 log C.U.

d Cultures assayed on 1×10^5 ^{51}Cr labelled B6.C-H-2^{ba} macrophages, incubation period, 16 hrs. Background = 4.80 log C.U.

e Cultures assayed on 1×10^5 ^{51}Cr labelled B6-H-2^{b} macrophages, incubation period, 16 hrs. Background = 4.80 log C.U.

f 4×10^6 irradiated B6.C-H-2^{ba} spleen stimulator cells were cultured with 2×10^6 B6-H-2^{b} lymph node responder cells for 5 days.

N.T. = not tested.

DISCUSSION

The use of a derived cytotoxicity unit has allowed comparative studies of the stimulator capacity of various lymphoid subpopulations and neoplastic cells of both lymphoid and non-lymphoid origin.

Lymphoid and non-lymphoid stimulators

All lymphoid tissues except the thymus and the peritoneum proved to be a rich source of stimulator cells. As will be shown in Chapter 5 the relatively poor stimulation observed with thymocytes reflects a deficiency of a necessary accessory cell population in the thymus rather than an innate inability of thymocytes to stimulate. A similar requirement for the addition of accessory cells when thymocytes are used as a source of stimulators has also been reported by others (Dyminski and Smith, 1975, 1976).

An alternate explanation must be invoked for the poor stimulation observed with peritoneal cells as this population is known to contain significant numbers of accessory cells (see Chapter 5). Since non-plastic adherent peritoneal cells induced significantly greater responses than unfractionated peritoneal cells at any of the concentrations tested, one possibility is that plastic adherent cells inhibit the response in some way. Support for this suggestion comes from recent reports where adherent peritoneal cells were shown to produce factors *in vitro*, capable of inhibiting cell division (Calderon *et al.*, 1974; Calderon and Unanue, 1975).

The characteristic dose response kinetics observed with neoplastic cells probably reflects the accumulation of toxic or inhibitory factors when these cells are used above a certain critical concentration. There is considerable evidence that some lymphomas and non-lymphoid neoplasias, such as P815 mastocytoma cells, can suppress both antibody and cell-mediated immune responses *in vitro* (Wong *et al.*, 1975; Tanapatchaiyapong and Zolla, 1974; Kamo *et al.*, 1975; Rodey *et al.*, 1974; Bonnard and Herberman, 1975;

Pikovski *et al.*, 1975). In some cases soluble factors have been shown to be responsible for the suppressive effects (Tanapatchaiyapong and Zolla, 1974; Pikovski *et al.*, 1975). The complete lack of stimulation at any concentration observed with IgG plasmacytoma cells suggests that this cell line may be a very efficient producer of suppressive agents.

In contrast to lymphocytes only small numbers of neoplastic cells were required to stimulate optimal responses. There may be a simple geometric explanation for this difference as neoplastic cells are considerably larger than lymphocytes. The possibility cannot be excluded, however, that the majority of neoplastic cells function as stimulators whereas only a subpopulation of lymphocytes may stimulate.

Adherence properties of stimulators

There is some controversy over the stimulator capacity of macrophage-like cells. While there are numerous reports of macrophages inducing proliferative responses in humans (Levis and Robbins, 1970; Bain and Lowenstein, 1969; Alter and Bach, 1970; Twomey *et al.*, 1970 and Rode and Gordon, 1974), mice (Talmage and Hemmingson, 1975; Schirrmacher *et al.*, 1975), and guinea pigs (Greineder and Rosenthal, 1974) few studies exist on the capacity of these cells to induce cytotoxic T cell responses (Wagner and Wyss, 1973; Simpson, 1975).

Two properties of macrophages, adherence to plastic surfaces and longevity in culture, were employed in the present study to separate macrophages from lymphocytes. The superior stimulation observed with splenic adherent cells as opposed to peritoneal adherent cells has also been reported by others. Thus Simpson (1975), showed that adherent spleen cells induced similar levels of cytotoxic activity to unfractionated spleen cells while Wagner and Wyss (1973) observed that adherent peritoneal cells induced considerably weaker responses than spleen cells. The reason for the differences in stimulator activity observed with splenic and peritoneal adherent cells is unknown but may reflect both functional and anatomical heterogeneity in adherent cell populations.

One disadvantage of using adherence properties to prepare macrophage-enriched populations is that cells other than macrophages may be adherent. That the adherent cells which stimulated in spleen were not all macrophages was well illustrated by the finding that Ig^-, θ^- cells (which include K cells, macrophages and null cells) induced 10-fold less cytotoxic activity than Ig^+, θ^+ cells.

When adherent cells were cultured for prolonged periods their capacity to stimulate was observed to decrease dramatically. This loss in stimulator activity did not appear to be a result of increased toxicity. A diminution in stimulator activity following prolonged culture of adherent cells has also been noted in proliferative studies in mice (Talmage and Hemmingson, 1975) and in humans (Rode and Gordon, 1974). However in other studies of proliferative responses with mouse (Schirrmacher *et al.*, 1975), human (Bain and Lowenstein, 1969; Twomey *et al.*, 1970) and guinea pig Greineder and Rosenthal, 1975) macrophages this phenomenon was not observed. At the moment, technical discrepancies and possibly variation between mouse strains can only be suggested as explanations for these differences.

In conclusion, it appears that adherent cells which stimulate cytotoxic T cell responses in the mouse strains tested here are not conventional long-lived, phagocytic, radiation-resistant macrophages but may be sticky lymphocytes; precursors of macrophages or perhaps conventional macrophages with rapidly lost absorbed antigens. Whatever the identity of these cells the spleen seems to be a richer source than the unstimulated peritoneal cavity. Unfortunately, to date, there are no reports which examine human or guinea pig macrophages as stimulators of cytotoxic T cell responses.

Surface markers on stimulator cells

In a recent study of murine lymphocytes it was suggested that the major antigenic stimulus recognised by cytotoxic T cell precursors is present predominantly on B cells (Simpson, 1975). In another study (Sondel *et al.*, 1975) however, and in this one, effective stimulation was observed with a

population depleted of both B cells and adherent cells (Wagner *et al.*, 1973; Sondel *et al.*, 1975).

The Ig^- population obtained by the rosetting procedure used in this study contains cell populations in addition to T cells (viz. macrophages, K cells and null cells). The relative contributions of each of these subpopulations to the stimulator activity of the whole population is technically difficult to determine. Although Ig^-, θ^- cells induced significant levels of cytotoxic activity and anti-θ and complement treatment of the Ig^- population had little effect on its stimulator activity (data not shown) at this stage it cannot be concluded that T cells are devoid of stimulator activity. The observation that carbonyl iron treated Ig^- populations which are considerably enriched for T cells are able to stimulate strong cytotoxic T cell responses in cultures supplemented with responder accessory cells strongly implies that T cells do in fact have the capacity to stimulate. The recent development in this laboratory of a theta (θ) rosetting procedure should help to resolve this issue.

Since adherent cell depleted Ig^+ and Ig^- populations both induced similar levels of cytotoxic activity when responder adherent cells were added in a three-cell system, the poorer stimulator observed with untreated Ig^- as opposed to untreated Ig^+ populations, may reflect the loss of an adherent accessory cell population during the separation. The loss of adherent cells, particularly when T cell enriched populations are prepared by filtration through nylon wool columns, could account for the inability reported by others (Simpson, 1975) of these cells to stimulate.

Separation of lymphocytes into subpopulations on the basis of the presence or absence of Fc and complement receptors, like separation on the basis of surface immunoglobulin, did not differentiate stimulator from non-stimulator populations.

As mentioned in Chapter 1, the central region of the H-2 complex, the I (immune response) region, codes for a group of polymorphic cell

surface structures designated Ia antigens (David *et al.*, 1973; Götze *et al.*, 1973; Hämmerling *et al.*, 1974). These antigens have been classed as strong LD antigens but weak target antigens, that is, they induce strong proliferative responses (Schendel *et al.*, 1973; Meo *et al.*, 1973b; Fathman *et al.*, 1974) but only weak cytotoxic T cell responses (Klein *et al.*, 1974b; Nabholz *et al.*, 1975b; Wagner *et al.*, 1975a). Recently there has been some debate as to whether the simultaneous recognition of Ia antigens or other LD antigens by a subset of collaborating T cells is a co-requisite for the generation of cytotoxic T cells (Schendel *et al.*, 1973, 1974; Wagner *al.*, 1973; Bach *et al.*, 1976).

The three experimental approaches described here, namely:

(a) the removal of Ia^+ cells from the stimulator population;

(b) the use of stimulator and responder populations sharing the I region and,

(c) the use of the mutant strain B6.C-H-2^{ba} and its congenic partner B6-H-2^{b} as stimulator and responder populations,

suggest that the presence of Ia^+ cells on even an I region difference is not an absolute requirement for the induction of cytotoxic T cell responses.

However, evidence was obtained which suggested that the recognition of determinants coded in the I region could result in the amplification of the response. Thus, the cytotoxic response obtained with mixtures of I region compatible CBA/H stimulator and A.TL responder cells could be significantly enhanced by the further addition of A.TH stimulator cells which differ only in the I region from the responder population. Wagner *et al.* (1976b) have recently obtained essentially similar results in the same system. Enhancement of proliferative and cytotoxic responses as a consequence of the presence of Ia^+ stimulator cells has also been reported (Cantor and Boyse 1975b; Wagner *et al.*, 1975b).

Since Mls locus coded antigens have been reported to replace Ia antigens as the LD requirement (Schendel and Bach, 1975), in the induction of cytotoxic T cells it may be argued that the stimulation observed with Mls different CBA/H and A.TL combinations resulted from the activation of T helper cells by Mls coded antigens. Although this cannot be excluded as a possibility the data obtained with mutant mice suggests that significant levels of cytotoxic activity can be generated in the absence of both I region differences and Mls locus differences. The reciprocal stimulation observed with mutant mice and their congenic partners is well documented (Klein *et al.*, 1975b; Forman and Klein, 1975; Melief *et al.*, 1975; Hodes *et al.*, 1976).

An alternate approach to the problem has been to separate the various T cell subpopulations responding in MLC and to determine the antigenic requirements for their induction. Recently Cantor and Boyse (1975a,b) have shown that Ly-1$^+$ helper cells responding primarily to Ia antigens can enhance the response of Ly-2,3$^+$ precursor cytotoxic T cells. It should be stressed, however, that significant amplification was only evident when Ly-2,3$^+$ cells were used in limiting numbers. Thus, optimal numbers of Ly-2,3$^+$ cells alone could generate substantial killer activity. Preliminary studies in this laboratory (data not shown) have indicated that the removal of Ly-1.1$^+$ T cells from the responding population has no effect on the ability of cytotoxic T cell precursors to respond.

To conclude, there is still considerable debate concerning the genetic requirements for induction of cytotoxic T cells. It would appear, though, that under optimal culture conditions the recognition of H-2K or H-2D region coded determinants by Ly-2,3$^+$ cells is sufficient for the generation of substantial cytotoxic activity. The response may be amplified by Ly-1$^+$ helper cells responding principally to antigens coded in the I region and possibly to other LD antigens. So far there is no evidence for the absolute requirement for T help that has been shown for the production of antibody to most antigens.

SUMMARY

The comparative ability of various lymphoid subpopulations and neoplastic cell lines to induce cytotoxic T cell responses *in vitro* was studied. Lymphocytes were observed to be the best source of stimulator cells followed by P815 mastocytoma cells, L cells and Ig A plasmacytoma cells. Mature macrophages were poor stimulators. Both Ig^- and Ig^+ cells were able to stimulate but optimal stimulation by Ig^- cells was found to be dependent on the addition of accessory cells. The presence of Fc or complement receptors or Ia antigens on lymphocytes did not confer superior stimulator capacity. The role of I region in the induction of cytotoxic T cell responses was also discussed.

CHAPTER 4

ADHERENT CELL REQUIREMENTS FOR CYTOTOXIC T CELL INDUCTION

4.1 Introduction

The requirement for a glass, nylon or cotton adherent accessory cell population for the induction of proliferative responses in MLC in human systems is well documented (Oppenheim *et al.*, 1968; Rode and Gordon, 1970; Twomey *et al.*, 1970; Alter and Bach, 1970; Berlinger *et al.*, 1976). These adherent cells may be of stimulator or responder origin (Rode and Gordon, 1970; Twomey *et al.*, 1970; Alter and Bach, 1970) and are thought to play an essential but non-specific role.

A similar requirement for glass or plastic adherent cells has also been reported for the generation of cytotoxic T cells in MLC in both murine systems (MacDonald *et al.*, 1973b; Wagner *et al.*, 1972), and in a xenogeneic rat/mouse system (Lonai and Feldman, 1971). To date there have been few attempts to characterise the functional adherent population or to determine its mode of action.

In this study the role of adherent cells in the generation of cytotoxic T cells was investigated by preincubating stimulator and responder populations on plastic or by pretreating them with carbonyl iron powder, a procedure known to remove conventional macrophages, other adherent subpopulations and non-viable cells (Lundgren *et al.*, 1968; Erb and Feldmann, 1975b).

4.2 Methods and materials

4.2.1 Animals

6-8 week old CBA/H, BALB/c, C57Bl/6, C57Bl/6 x BALB/c F_1 and BALB/c x CBA/H F_1 mice were used routinely.

4.2.2 Tissue culture procedures

Cells were prepared and cultured as described in Chapters 2 and 3 (sections 2.2.3 and 3.2.2).

4.2.3 Cytotoxicity assays

Cultures were assayed for cytotoxic activity as described in Chapter 2 (section 2.2.5).

4.2.4 Removal of adherent populations

Adherent cells were removed by incubation on plastic surfaces or by treatment with carbonyl iron powder as described in Chapter 3 (section 3.2.4).

4.2.5 Trypsin treatment of carbonyl iron residues

Cells were treated with carbonyl iron powder as described in Chapter 2. Following the removal of the non-adherent cells the carbonyl iron residue was washed twice in serum free medium. All supernatants were collected and added back to the original depleted population. The carbonyl iron residue was then resuspended in serum free medium containing trypsin at a concentration of 1 mg/ml. The suspension was mixed for 30' at 37°C then cold medium containing foetal calf serum was added. The carbonyl iron was removed and the residue washed once. All supernatants were added back to the original depleted population. The cells were incubated for 2 hours at 37°C prior to irradiation.

4.3 Results

4.3.1 The effects of removal of adherent cells from stimulator and responder populations

A. Lymph node responder cells - BALB/c spleen stimulator cells and CBA/H lymph node responder cells were either treated with carbonyl iron powder or incubated on plastic petri dishes to remove adherent cells. Following treatment with carbonyl iron powder 15-25% of spleen cells and 10-15% of lymph node cells were routinely removed. Similar proportions of cells were also lost following incubation on plastic. 4×10^6 spleen cells and 2×10^6 lymph node cells were used routinely. The concentration of treated populations was adjusted according to the proportion of cells lost (thus if 20% of spleen cells were lost following treatment the cell concentration was adjusted to 3.2×10^6/ml).

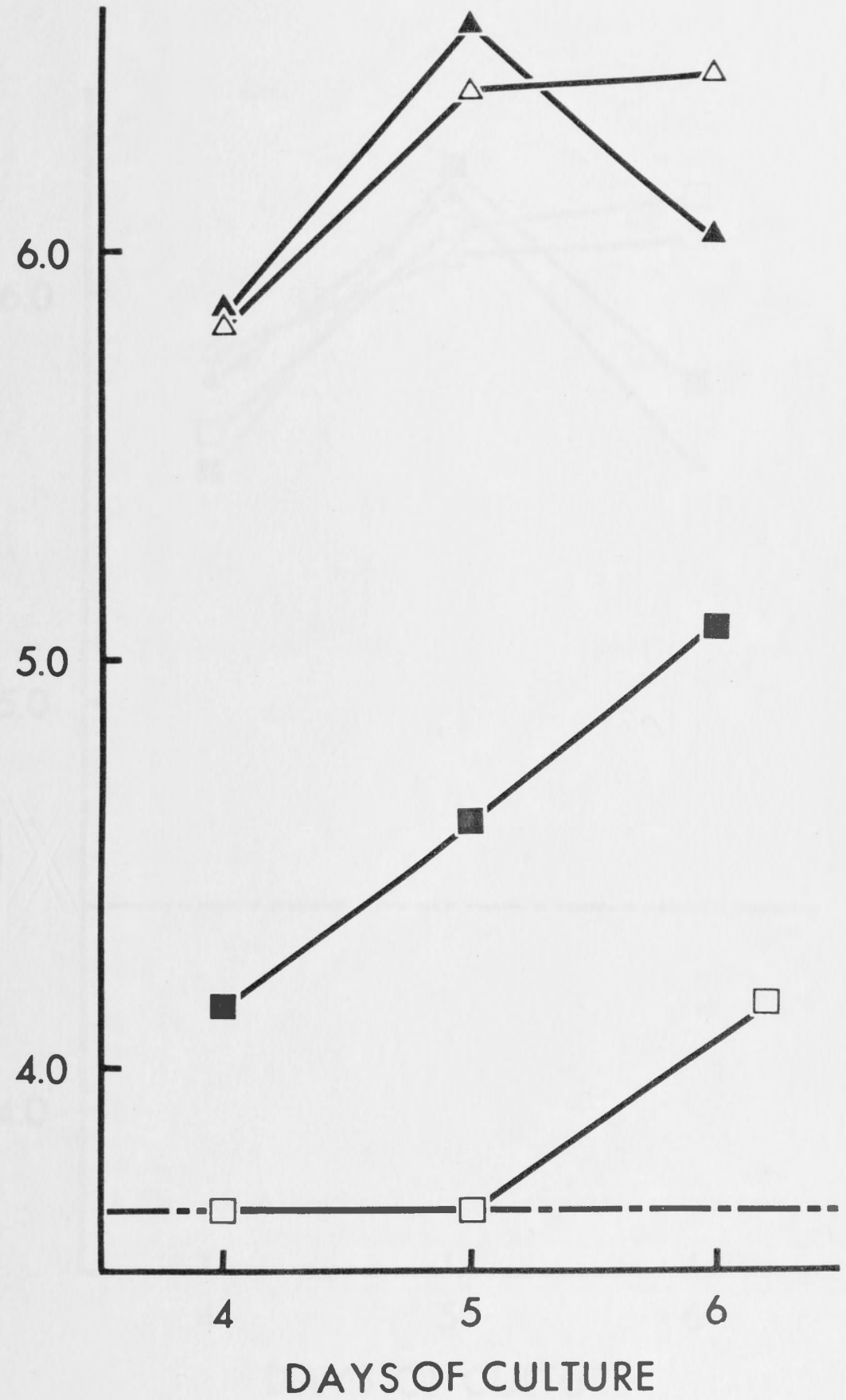

Figure 4.1 - The effects of carbonyl iron treatment of spleen stimulator cells and lymph node responder cells on the generation of cytotoxic activity.

Stimulator = BALB/c spleen cells; responder = CBA/H lymph node cells; treatment = carbonyl iron treatment prior to culture.

▲—▲, treatment of responders only; △—△, no treatment of stimulator or responder; ■—■, treatment of stimulator only; □—□, treatment of both stimulator and responder.

▬ ▬ ▬ = background value.

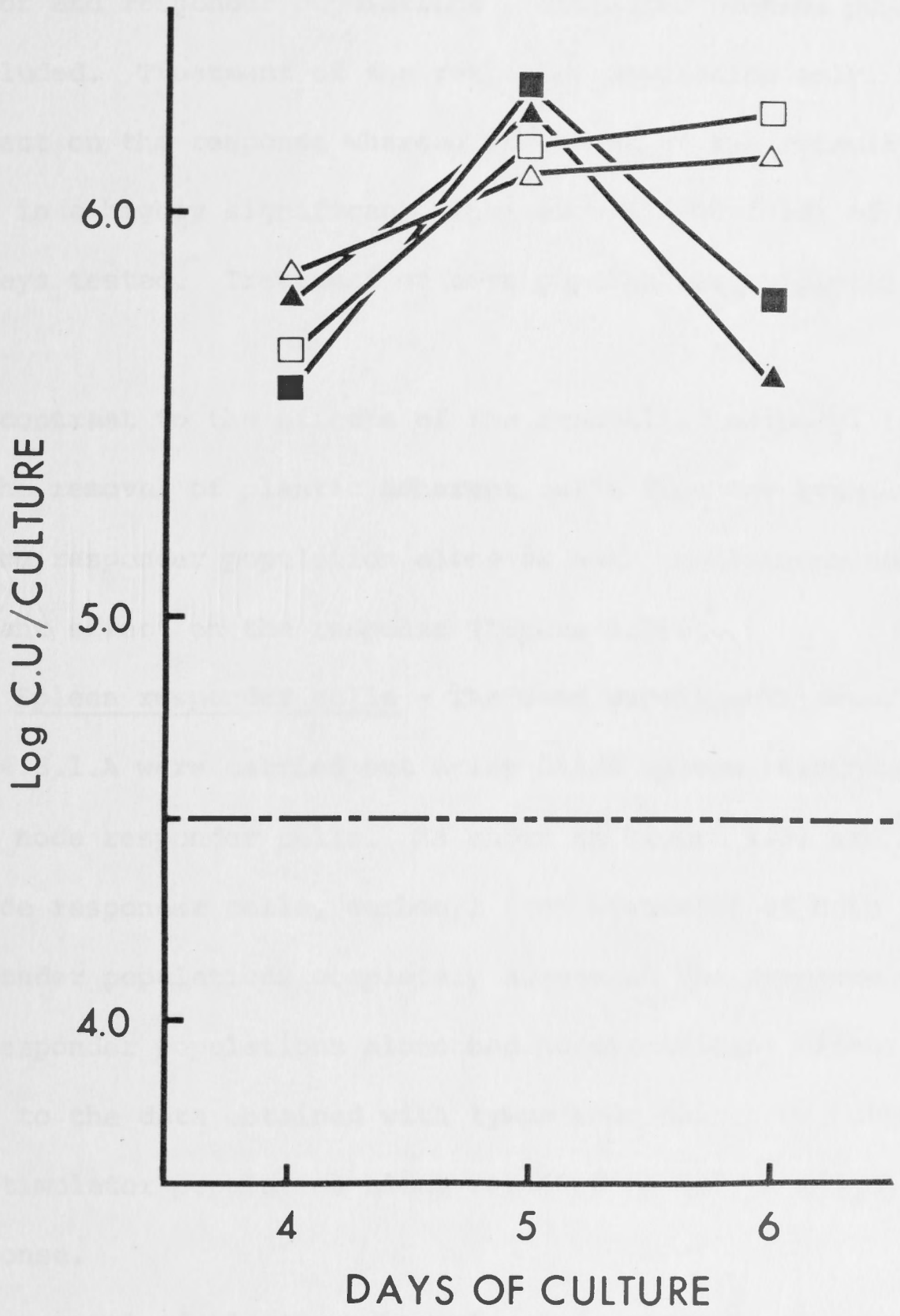

Figure 4.2 - The effects of the removal of plastic adherent cells from stimulator and responder populations on the generation of cytotoxic activity.

Treatment = incubation on plastic for 4 hrs prior to culture.

Stimulator = BALB/c spleen cells; responder = CBA/H lymph node cells.

▲—▲, treatment of responder only; △—△, no treatment of responder or stimulator; ■—■, treatment of stimulator only; □—□, treatment of both stimulator and responder.

▬ ▬ ▬ = background value.

Figure 4.1 shows the effects of carbonyl iron treatment of the responder population alone, the stimulator population alone and both stimulator and responder populations. Untreated control populations were also included. Treatment of the responder population only, had no significant effect on the response whereas treatment of the stimulator population resulted in a highly significant depression (20-50 fold) of the response on all days tested. Treatment of both populations completely abrogated the response.

In contrast to the effects of the removal of carbonyl iron adherent cells, the removal of plastic adherent cells from the stimulator population alone, the responder population alone or both populations together had no significant effect on the response (Figure 4.2).

B. Spleen responder cells - The same experiments described in section 4.3.1.A were carried out using CBA/H spleen responder cells instead of lymph node responder cells. As shown in Figure 4.3, and as observed with lymph node responder cells, carbonyl iron treatment of both the stimulator and responder populations completely abrogated the response while treatment of the responder populations alone had no significant effect. However, in contrast to the data obtained with lymph node cells, carbonyl iron treatment of the stimulator population alone resulted in only a slight reduction in the response.

The removal of plastic adherent cells, prior to culture, from either the responder or the stimulator population or both, had no significant effect on the response (Figure 4.4).

4.3.2 Some aspects of carbonyl iron treatment

The data presented in Figures 4.1 - 4.4 suggest that a population of cells is required for the induction of cytotoxic T cells which is:

(a) depleted by treatment with carbonyl iron but not by incubation on plastic surfaces,

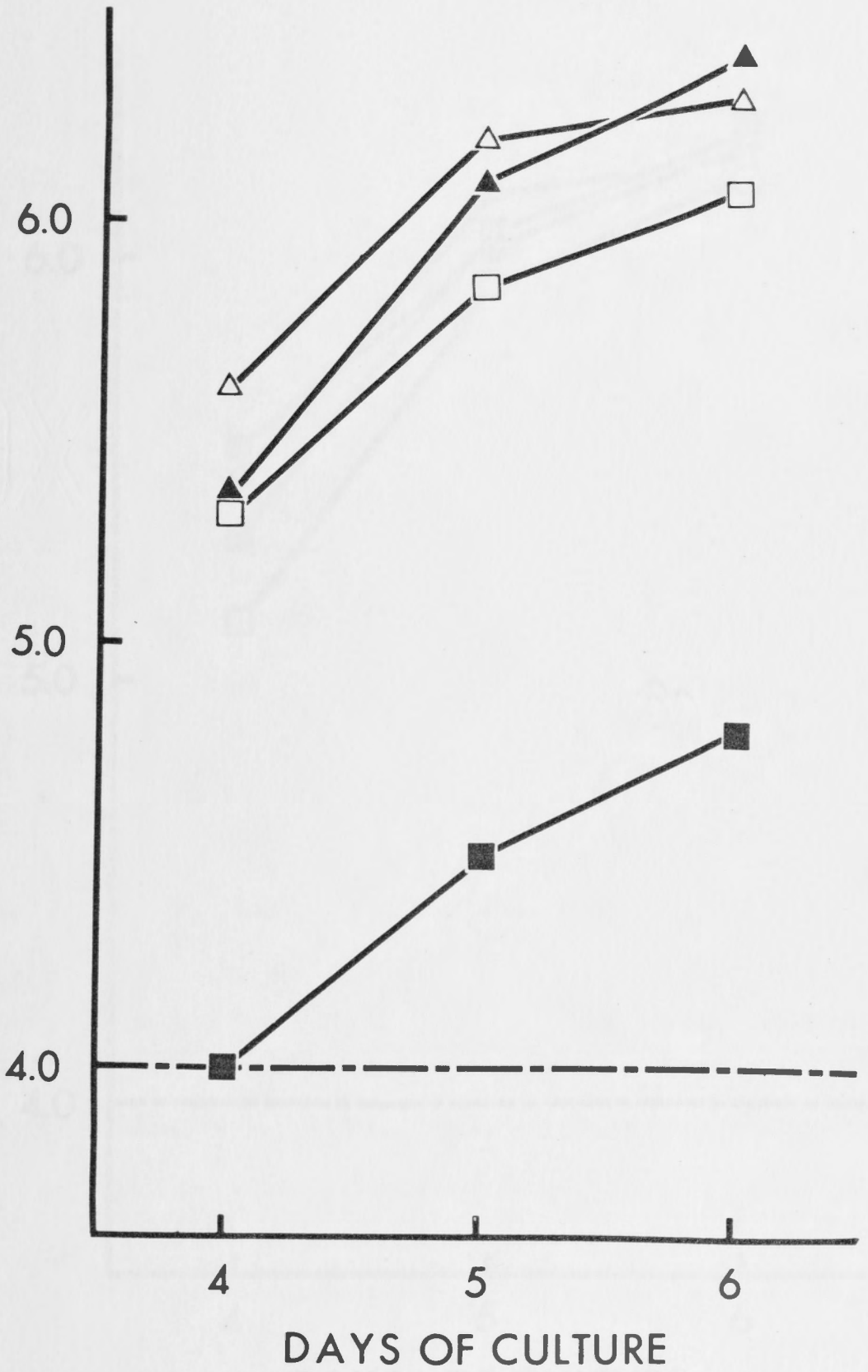

Figure 4.3 - The effects of carbonyl iron treatment of spleen stimulator cells and spleen responder cells on the generation of cytotoxic activity. Treatment = treatment with carbonyl iron prior to culture; stimulator = BALB/c spleen cells; responder = CBA/H spleen cells. ▲—▲ , treatment of responder only; △—△ , no treatment of responder or stimulator; ■—■ , treatment of both responder and stimulator; □—□ , treatment of stimulator only. ▬ ▬ ▬ = background value

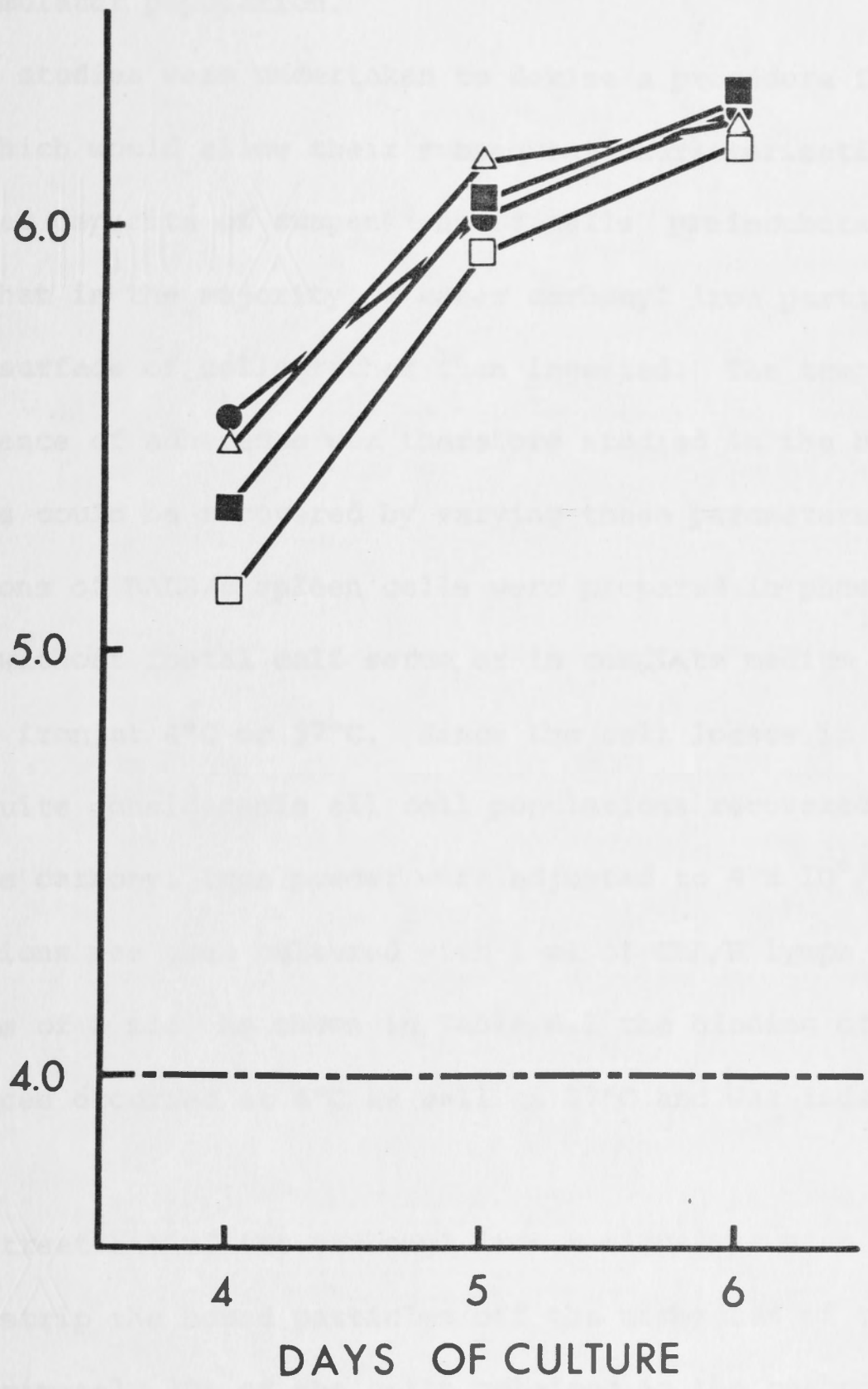

Figure 4.4 - The effects of the removal of plastic adherent cells from spleen stimulator and spleen responder populations on the generation of cytotoxic activity.

Stimulator = BALB/c spleen cells; responder = CBA/H spleen cells; treatment = incubation on plastic for 4 hrs prior to culture.

△—△, treatment of responder only; ■—■, treatment of stimulator only; ●—●, no treatment of stimulator or responder; □—□ treatment of both stimulator and responder. ▬ ▬ ▬, background value.

(b) present predominantly in spleen and

(c) effective whether syngeneic with the responder or the stimulator population.

The following studies were undertaken to devise a procedure for recovering these cells which would allow their subsequent characterisation. Stained cytocentrifuged deposits of suspensions of cells preincubated with carbonyl iron showed that in the majority of cases carbonyl iron particles were bound to the surface of cells rather than ingested. The temperature and cation dependence of adherence was therefore studied in the hope that the adherent cells could be recovered by varying these parameters.

Suspensions of BALB/c spleen cells were prepared in phosphate buffered saline (PBS) without foetal calf serum or in complete medium and were mixed with carbonyl iron at 4°C or 37°C. Since the cell losses in serum free medium were quite considerable all cell populations recovered following removal of the carbonyl iron powder were adjusted to 4×10^6/ml. 1 ml of these suspensions was then cultured with 1 ml of CBA/H lymph node cells in a final volume of 2 ml. As shown in Table 4.1 the binding of carbonyl iron to cell surfaces occurred at 4°C as well as 37°C and was independent of calcium ions.

Trypsin treatment of the carbonyl iron residue was also attempted in an effort to strip the bound particles off the membranes of the accessory cells. Approximately 20% of the cells retained in the carbonyl iron were released by this treatment, but as shown in Table 4.1, addition of this population to the depleted population did not reconstitute the response.

There are two lines of evidence which indicate that the carbonyl iron is not merely toxic for unbound cells. First, carbonyl iron treatment of the responder population alone had no effect on the response and second, responder spleen cells could reconstitute the response to treated stimulator cells. Further evidence that the capacity to stimulate or to respond is not affected following carbonyl iron treatment comes from studies with P815

TABLE 4.1

THE EFFECTS OF TEMPERATURE AND Ca^{++} CONCENTRATION ON DEPLETION OF ADHERENT CELLS BY CARBONYL IRON TREATMENT

Carbonyl iron treatment	Other treatments	Cytotoxic activity log C.U./culture
-[c]	-	5.85[a]
+	-	4.50
+	Calcium free medium, 4°C	Bg[b]
+	Calcium free medium, 37°C	Bg
+	Complete medium, 4°C	Bg
+	Trysin treatment of carbonyl iron residue	Bg

a 4×10^6 irradiated BALB/c spleen stimulator cells and 2×10^6 CBA/H lymph node responder cells were used throughout. Cultures were assayed on day 5 on ^{51}Cr-labelled P815 targets.

b Bg = <4.50 log C.U.s.

c - = not treated; + = treated.

stimulator cells and CBA/H lymph node responder cells. As Table 4.2 shows carbonyl iron treatment of P815 stimulator cells has no significant effect on the response.

4.3.3 Does removal of adherent cells from mixed lymphocyte cultures result in selective generation of suppressor cells or paralysis of the responder population?

One explanation for the abrogation of the cytotoxic response following the removal of carbonyl iron adherent cells from both stimulator and responder populations is that such a treatment allows the selective generation of suppressor T cells.

Alternately, the lack of response may reflect a tolerance phenomenon. Thus adherent cells may provide, either directly or indirectly, a signal necessary for the induction of cytotoxic T cell precursors. In the absence of this signal but in the presence of antigen cytotoxic T cell precursors may become tolerised.

An experimental system was designed whereby both of these possibilities could be tested simultaneously. C57Bl/6 x BALB/c F_1 stimulator spleen cells and C57Bl/6 lymph node responder cells were both treated with carbonyl iron. The F_1 spleen cells were then irradiated and cultured with either 2×10^6 or 4×10^6 C57Bl/6 lymph node cells. Cultures were incubated for 48 hours then 4×10^6 irradiated CBA/H x BALB/c F_1 spleen cells were added to each culture. Control cultures consisting of 4×10^6 CBA/H x BALB/c F_1 and BALB/c x C57Bl/6 F_1 irradiated spleen cells and 4×10^6 or 2×10^6 C57Bl/6 lymph node cells were also set up. Cultures were incubated for 5 days and then assayed on P815 or L cell targets. As the data in Table 4.3 shows, preincubation of C57Bl/6 responder cells in the presence of antigen and the absence of adherent cells, had no effect on their ability to subsequently generate a response to either $\underline{H\text{-}2}^k$ or $\underline{H\text{-}2}^d$ coded antigens. Thus the removal of adherent cells resulted in neither the paralysis of the responder population nor the selective generation of suppressor cells.

TABLE 4.2

CARBONYL IRON TREATMENT OF P815-X2 MASTOCYTOMA STIMULATOR CELLS

Stimulator population	Cytotoxic activity log C.U./culture
Untreated P815 cells[a]	5.67[b]
Carbonyl iron treated P815 cells	5.87

a 1×10^5 irradiated (5000 R) P815 stimulators and 2×10^6 CBA/H lymph node cells used. Cultures assayed on ^{51}Cr labelled P815 targets on day 5.

b Background = 4.65 log C.U.

TABLE 4.3

THE EFFECTS OF PREINCUBATION OF RESPONDER CELLS IN THE PRESENCE OF ANTIGEN AND THE ABSENCE OF ADHERENT CELLS

Concentration of responders	Pretreatment of stimulator and responder populations	Cytotoxic activity log C.U./culture	
		P815 targets	L cell targets
4×10^6	+[a]	6.20	5.21
2×10^6	+[a]	6.36	5.45
4×10^6	+[b]	Bg[d]	Bg[e]
4×10^6	-[c]	6.20	5.18
2×10^6	-[c]	6.33	5.44

a + = treated populations.
4×10^6 irradiated carbonyl iron treated BALB/c x C57Bl/6 F_1 spleen cells were cultured with 4×10^6 or 2×10^6 C57Bl/6 carbonyl iron treated lymph node cells. After 48 hrs, 4×10^6 irradiated BALB/c x CBA/H F_1 spleen cells were added. Cultures were assayed 5 days later.

b Same treatment as (a) but no BALB/c x CBA/H F_1 cells were added after 48 hrs.

c - = untreated populations.
Control cultures set up on day -5 consisting of 4×10^6 irradiated BALB/c x CBA/H F_1 spleen cells plus 4×10^6 irradiated BALB/c x C57Bl/6 F_1 spleen cells and C57Bl/6 lymph node responder cells.

d Background (Bg) = 4.73 log C.U.

e Background (Bg) = 4.23 log C.U.

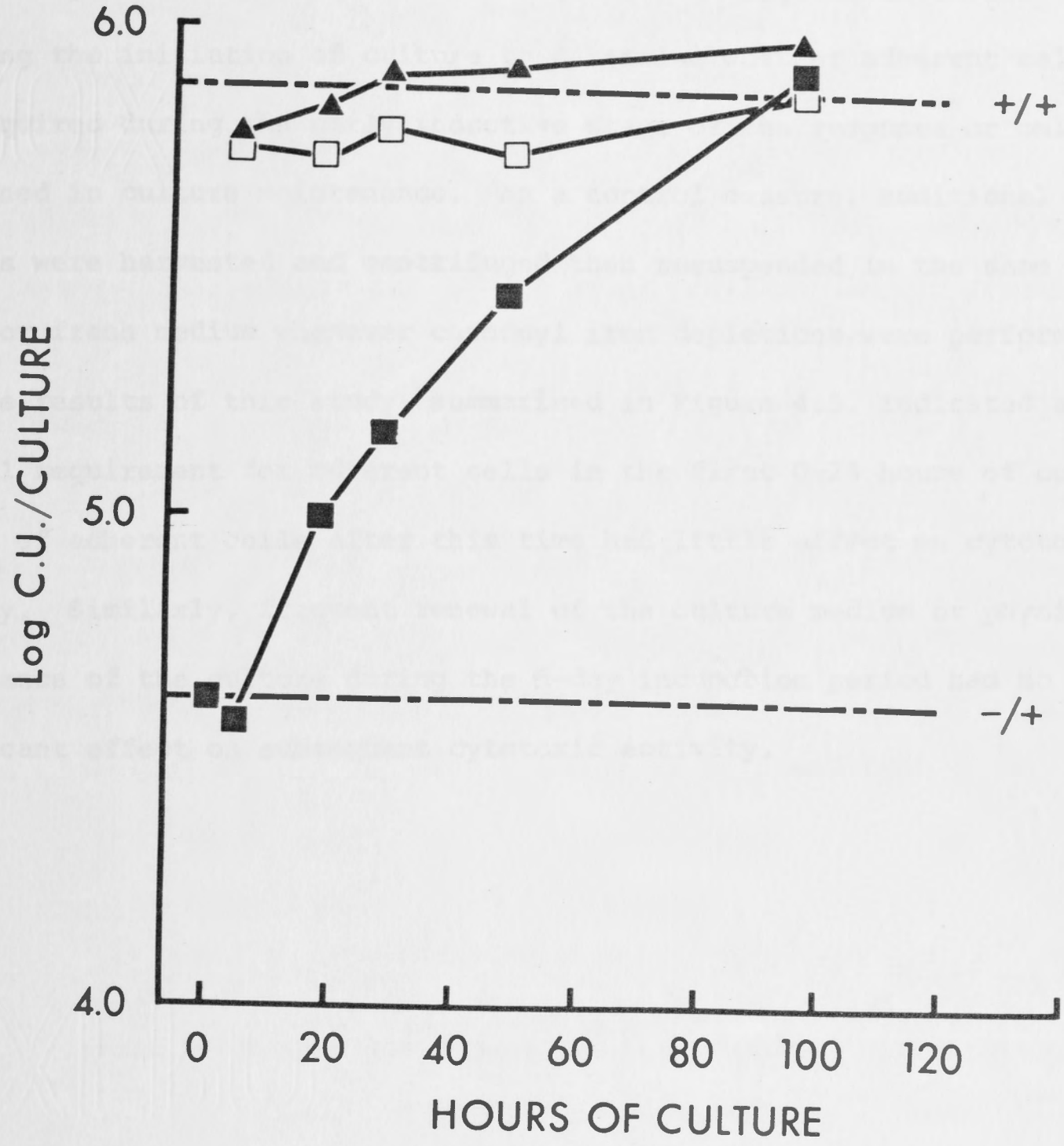

Figure 4.5 - The effects of the removal of accessory cells at various times during the culture period.

Stimulator = BALB/c spleen cells; responder = CBA/H lymph note; treatment = treatment with carbonyl iron.

+/+, cytotoxic activity generated with untreated stimulator and responder populations

-/+, cytotoxic activity generated with treated stimulator and untreated responder populations.

■—■, treatment of cultures at the time points specified.

□—□, addition of fresh medium at the time points specified.

△—△, agitation of the cultures at the time points specified.

4.3.4 Time course for the removal of carbonyl iron adherent cells

Carbonyl iron depletions were carried out at regular intervals following the initiation of culture to determine whether adherent cells were required during the early inductive stage of the response or only functioned in culture maintenance. As a control measure, additional cultures were harvested and centrifuged then resuspended in the same medium or fresh medium whenever carbonyl iron depletions were performed.

The results of this study, summarised in Figure 4.5, indicated a critical requirement for adherent cells in the first 0-24 hours of culture. Removal of adherent cells after this time had little effect on cytotoxic activity. Similarly, frequent renewal of the culture medium or physical disturbance of the culture during the 5-day incubation period had no significant effect on subsequent cytotoxic activity.

DISCUSSION

The results reported in this chapter suggest that when lymphocytes are used as stimulator cells in mixed lymphocyte cultures there is an additional and absolute requirement for an adherent accessory cell population. This population is very efficiently depleted by treatment with carbonyl iron but, in contrast to reports by MacDonald *et al.* (1973b) does not bind avidly to plastic.

Although the accessory cell populations required for the induction of cytotoxic T cell responses are removed by methods conventionally used for the depletion of macrophages the conclusion that these accessory cells, like those required for the induction of antibody responses *in vitro* are macrophages should not be hastily drawn.

The observation that the removal of the accessory cells required for the induction of cytotoxic T cells is not temperature, serum or calcium ion dependent suggests that the active cells are not conventional macrophages. Shortman and others (1972) have shown that in addition to macrophages and polymorphs, dead cells and low density cells including immature and dividing cells of both T and B cell lineage adhere to glass bead columns. In addition, adherence of dead cells and low density cells, unlike that of macrophages and polymorphs, was temperature independent. A similar population of "sticky" low density lymphocytes may also bind carbonyl iron. The very efficient removal of dead cells and, in one report, B cells (Erb and Feldmann, 1975b) by carbonyl iron suggests that "sticky" cells can indeed be effectively removed by this method. Thus, the possibility cannot be excluded that the accessory cells active in the induction of cytotoxic T cells are included in the temperature independent "sticky" lymphocyte subpopulation while those involved in antibody formation are members of the temperature dependent macrophage-like population.

The accessory cells depleted by carbonyl iron treatment may be of either stimulator or responder origin and appear to be present in greater concentration in spleen than in lymph node cell preparations. The former observation has also been made by others in studies of both proliferative (Rode and Gordon, 1970; Twomey *et al.*, 1970; Alter and Bach, 1970) and cytotoxic T cell (MacDonald *et al.*, 1973b) responses. Two ways by which accessory cells may function in MLR include antigen processing or presentation and culture maintenance.

It is well documented that a macrophage-like adherent cell population is required for the induction of antibody responses to most antigens (for review see Pierce and Kapp, 1976). Recent studies have suggested that for primary responses to T cell dependent antigens the adherent cells may be either syngeneic or allogeneic with the responding helper T cells (Pierce *et al.*, 1976). For a secondary response, however, the antigen must be presented on macrophages syngeneic to those used for the primary immunisation (Rosenthal and Shevach, 1973; Pierce *et al.*, 1976). This restriction in response was found to be controlled by the MHC. One interpretation of these observations is that antigen is presented on the surface of adherent cells in unique association with antigens coded by the MHC (probably the I region). Once sensitised to any particular complex a cell will only be restimulated by the same complex.

It seems unlikely that the accessory cells required for the generation of cytotoxic T cells function in the same way as those involved in antibody formation since the presentation of soluble stimulator MHC antigens on the surface of responder adherent cells would require that responder MHC antigens, as well as stimulator MHC antigens, be recognised by the effector population. The fact that subcellular antigen fractions do not stimulate primary cytotoxic T cell responses (Wagner and Boyle, 1972; Engers *et al.*, 1975b; Häyry and Andersson, 1976; Wagner *et al.*, 1976a).

provides further evidence against the involvement of accessory cells in antigen processing or presentation.

If accessory cells are not involved in antigen presentation it is possible that they merely optimise tissue culture conditions. Although culture maintenance cannot be excluded as a secondary function of accessory cells their critical early requirement in cultures (the first 24 hours) suggests that they have a more important primary role in the induction or recognitive phase of the MLR. Possible mechanisms by which accessory cells may function in the induction of cytotoxic T cells will be discussed in more detail in subsequent chapters.

In addition to demonstrating the requirement for accessory cells in MLR the studies reported in this chapter have also convincingly shown that purified lymphocytes are very efficient stimulators provided they are cultured in the presence of an adequate number of accessory cells. Thus mice and humans seem to differ from guinea pigs in this respect, since guinea pig lymphocytes, even in the presence of responder accessory cells, are poor stimulators in MLC (Greineder and Rosenthal, 1975).

That the abrogation of the response following removal of accessory cells from both the stimulator and responder populations was not a result of the selective generation of suppressor cells or to tolerisation of the responder population was also shown. Attempts to paralyse responder cells by incubating them with stimulator lymphocytes in the absence of 2-ME and accessory cells (data not shown) were also unsuccessful. Before dismissing a 2-signal model of induction similar to that proposed by Bretscher and Cohn (1970) for the induction of B cells, where recognition of antigen in the absence of an inductive signal results in paralysis of the responder population, the possibility that extraneous sources of signal-2 sufficient for the prevention of paralysis but insufficient for induction, would have to be eliminated.

S U M M A R Y

The data presented in this chapter indicated that when resting lymphocytes were used a source of stimulator cells in MLC there was an additional and absolute requirement for a population of accessory cells which was removed by treatment with carbonyl iron but not by incubation on plastic surfaces. This adherent population was present predominantly in spleen and was effective whether syngeneic with the responder or the stimulator population.

The accessory cells were only required in the first 24 hours of culture, a result which implied that they were actively involved in the inductive process and did not merely function in culture maintenance. Furthermore, indirect evidence suggested that the active cells were not mature macrophages and were unlikely to function in antigen presentation.

Carbonyl iron treatment did not result in the selective generation of suppressor cells or paralysis of the responder population. The failure to recover functional accessory cells from carbonyl iron residues suggested that the cells may have been destroyed during the depletion process.

CHAPTER 5

CHARACTERISATION OF ACCESSORY CELLS

5.1 Introduction

In the previous chapter it was shown that when resting lymphocytes were used as a source of stimulator cells there was an additional and absolute requirement for an accessory cell population which did not bind avidly to plastic but which was very efficiently removed by treatment with carbonyl iron. Since the active cell population could not be recovered from the carbonyl iron residue an alternative method had to be contrived for its characterisation.

An assay system was devised which took advantage of the finding that accessory cells could be of either stimulator or responder origin. It consisted of a "three-cell system" which was comprised of irradiated carbonyl iron treated stimulator cells, untreated lymph node responder cells and irradiated accessory cell- containing populations of responder genotype. Using such a system the accessory cell activity of various lymphoid populations was evaluated.

In addition, the accessory cell requirements for primary responses to neoplastic cells and for secondary responses to lymphoid cells were also assessed. The accessory cell requirements for the restimulation of memory cells was of particular interest since it has been well documented that the requirements for restimulation are much less stringent than for primary induction. Thus UV treated (Röllinghoff and Wagner, 1975; Häyry and Andersson, 1976), heat treated or glutaraldehyde fixed (Röllinghoff and Wagner, 1975), stimulator cells and subcellular fragments (Wagner *et al.*, 1976a; Hayry and Andersson, 1976) have all been reported to stimulate secondary responses but not primary responses.

5.2 Methods and Materials

5.2.1 Animals

8-10 week old CBA/H and BALB/c mice were used routinely.

5.2.2 Tissue culture procedures

Cells were prepared as described in Chapter 2 (2.2.3) and Chapter 3 (3.2.8). Mixed lymphocyte cultures and cytotoxicity assays were performed as described in Chapter 2, sections 2.2.4 and 2.2.5 and Chapter 3 (3.2.2).

5.2.3 Carbonyl iron treatment

Cells were treated with carbonyl iron as described in Chapter 3 (3.2.4).

5.2.4 Removal of Ig^+, Ia^+ and θ^+ cells

The procedures used for the removal of these cells are outlined in sections 3.2.9; 3.2.7 and 3.2.6, respectively, of Chapter 3.

5.2.5 Preparation of adherent cell monolayers

Spleen and peritoneal adherent cell monolayers were prepared as described in Chapter 3 (3.2.8). Macrophage monolayers were prepared in a similar way but were cultured for three days prior to use.

5.2.6 Neoplastic cells

P815-X2 DBA/2 mastocytoma cells and L-929 cells were pretreated as outlined in Chapter 3 (3.2.10) prior to use as stimulator cells.

5.2.7 Immunisation of mice with P815 cells

20 µl of washed, packed P815 cells ($\sim 2 \times 10^7$) were injected into the footpads of CBA/H mice. A sample of popliteal lymph nodes was taken 10-14 days later and tested for cytotoxic activity. The remainder of the animals were left for 6-8 weeks before use. In all cases only popliteal lymph nodes were used as a source of primed lymph node responder cells. Unimmunised animals of the same age were used as controls.

TABLE 5.1

ADDITION OF CBA SPLEEN ACCESSORY CELLS RESTORES THE RESPONSE TO CARBONYL IRON TREATED POPULATIONS

Stimulator population	Carbonyl iron treatment[a]	Accessory cell population[b]	Carbonyl iron treatment	Cytotoxic activity log C.U./culture
4×10^6 BALB/c spleen cells	-	-	-	5.76[c]
"	+	-	-	4.02
"	+	4×10^6 CBA/H spleen	-	5.76
"	+	2×10^6 CBA/H spleen	-	5.90
"	+	1×10^6 CBA/H spleen	-	5.15
"	+	4×10^6 CBA/H spleen	+	4.02
"	-	4×10^6 CBA/H spleen	-	4.02[d]

a Carbonyl iron treatment removed 29% of cells.

b CBA/H spleen accessory cells were irradiated (1000 R).

c 2×10^6 CBA/H lymph node responder cells were used except where otherwise stated.

d 4×10^6 irradiated BALB/c spleen cells were cultured with 4×10^6 irradiated CBA/H spleen cells in the absence of responder cells.

+ = treatment; - = no treatment. Background = 4.02 log C.U.

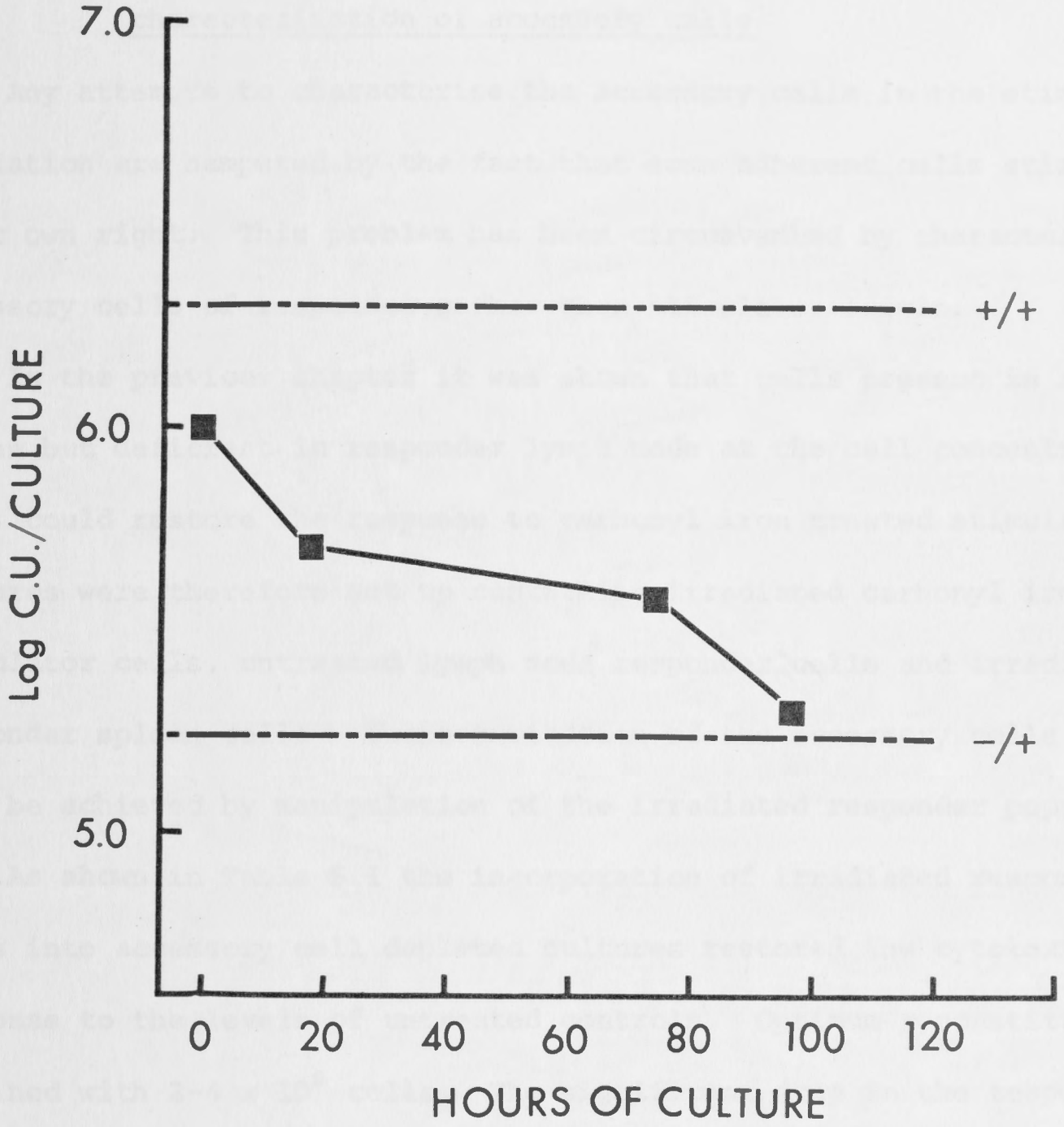

Figure 5.1 - Time course for the addition of accessory cells. ■——■, 4×10^6 irradiated CBA/H spleen cells were added to cultures of irradiated carbonyl iron treated BALB/c spleen cells and 2×10^6 CBA/H lymph node responder cells at the specified times. Cultures were assayed after 5 days on ^{51}Cr labeled P815 targets.

+/+, Cytotoxic activity generated in cultures of untreated BALB/c spleen stimulator cells and CBA/H lymph node responder cells.

-/+, Cytotoxic activity generated in cultures of carbonyl iron treated BALB/c spleen cells and CBA/H lymph node responder cells.

5.3 Results

5.3.1 Description of the three-cell system used for the characterisation of accessory cells

Any attempts to characterise the accessory cells in the stimulator population are hampered by the fact that some adherent cells stimulate in their own right. This problem has been circumvented by characterising accessory cells of responder rather than stimulator origin.

In the previous chapter it was shown that cells present in responder spleen but deficient in responder lymph node at the cell concentrations used, could restore the response to carbonyl iron treated stimulator cells. Cultures were therefore set up containing irradiated carbonyl iron treated stimulator cells, untreated lymph node responder cells and irradiated responder spleen cells. Characterisation of the accessory cells could then be achieved by manipulation of the irradiated responder population.

As shown in Table 5.1 the incorporation of irradiated responder spleen cells into accessory cell depleted cultures restored the cytotoxic response to the levels of untreated controls. Optimum reconstitution was attained with 2-4 x 10^6 cells. The significant drop in the response when only 1 x 10^6 cells were used suggests that a critical number of accessory cells must be present before a response is induced.

That restoration of the response resulted from the addition of adherent cells was evidenced by the abrogation of restorative capacity following carbonyl iron treatment of the irradiated CBA/H spleen cells (Table 5.1). Furthermore, since combinations of irradiated BALB/c and irradiated CBA/H spleen cells do not generate effector cells it is unlikely that cells in the irradiated CBA/H spleen population contribute directly to the effector population.

The results of experiments involving the addition of irradiated CBA/H spleen cells to accessory cell depleted cell mixtures at various time intervals after the initiation of culture are illustrated in Figure 5.1.

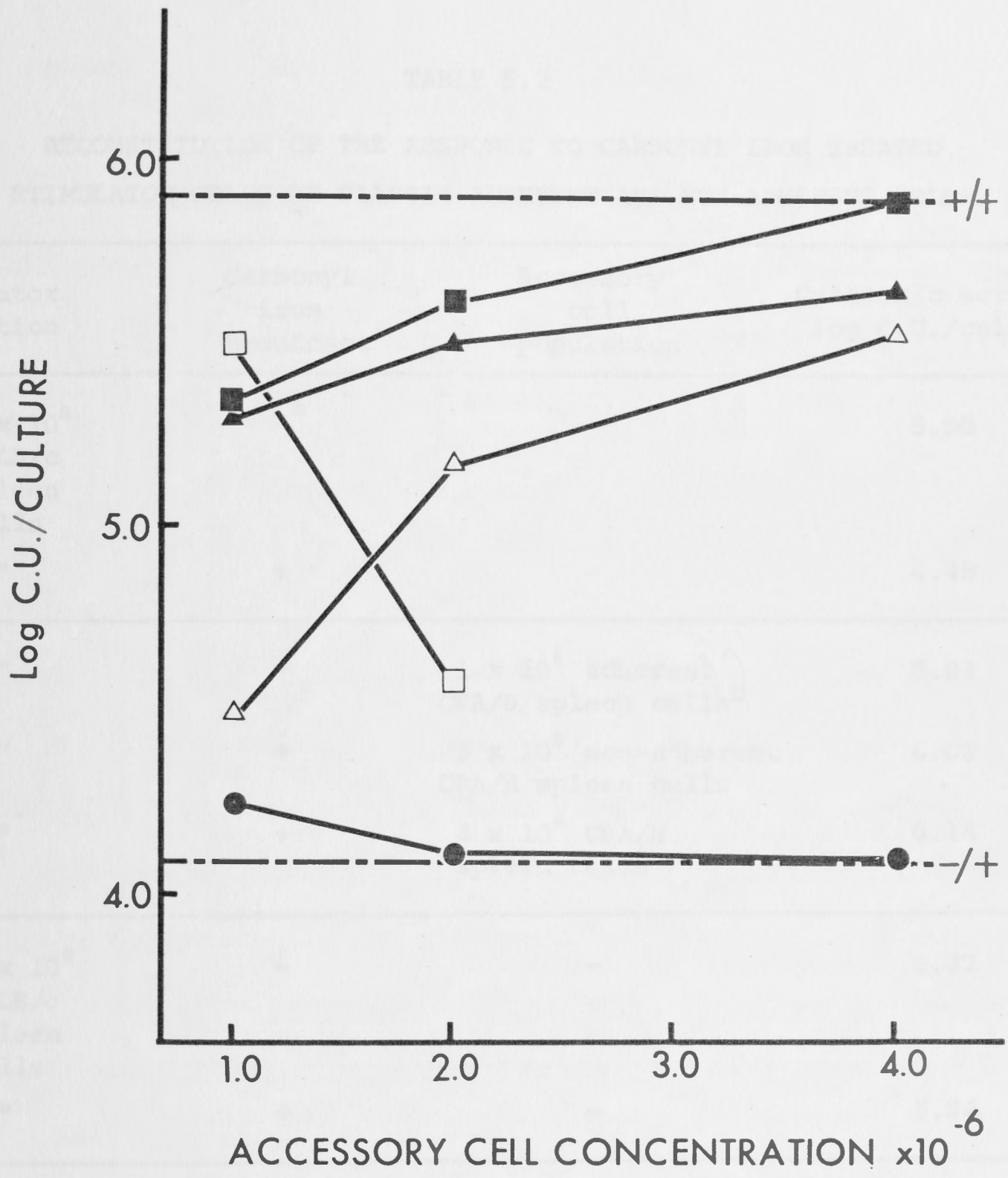

Figure 5.2 - Accessory cell content of various lymphoid tissues.

+/+, Culture containing 4 x 10^6 irradiated BALB/c spleen cells and 2 x 10^6 CBA/H lymph node cells.

-/+, Culture containing 4 x 10^6 irradiated carbonyl iron treated BALB/c spleen cells and 2 x 10^6 CBA/H lymph node cells.

Effects of addition irradiated CBA/H cells to (-/+) cultures.

■ , spleen; △ , lymph node; ▲ , bone marrow;

□ , peritoneal cells; ● , thymus.

TABLE 5.2

RECONSTITUTION OF THE RESPONSE TO CARBONYL IRON TREATED STIMULATOR CELLS BY PLASTIC ADHERENT AND NON-ADHERENT CELLS

	Stimulator population	Carbonyl iron treatment	Accessory cell population	Cytotoxic activity log C.U./culture[d]
A.	4×10^6 BALB/c spleen cells	-[a]	-	5.90
	"	+	-	4.48
	"	+	1×10^6 adherent CBA/H spleen cells[b]	6.03
	"	+	3×10^6 non-adherent CBA/H spleen cells	6.03
	"	+	4×10^6 CBA/H spleen cells	6.14
B.	4×10^6 BALB/c spleen cells	-	-	6.32
	"	+	-	5.24
	"	+	2×10^5 BALB/c adherent P.C.	6.06[c]
	"	+	1×10^5 CBA/H adherent P.C.	5.85
	"	+	2×10^5 BALB/c	Bg[e]

a + = treatment; - = no treatment.

b Adherent cells were those which adhered to plastic in 4 hrs at 37°C. Non-adhernet cells were those cells removed following vigorous washing of the adherent cell monolayers. All splenic accessory cells were irradiated (1000 R).

c The cytotoxic activity generated by BALB/c adherent peritoneal cells was subtracted from this value.

d Cytotoxic activity was assayed on ^{51}Cr labeled P815 cells in a 4 hr assay.

e Background = 4.00 log C.U.

It can be seen that the accessory cell population is required during the first 24 hours of culture, a finding which confirms the results obtained from depletion experiments described in the previous chapter where accessory cells were removed from the cell mixtures at regular time intervals after the initiation of culture (compare Figures 4.5 and 5.1). These experiments thus provided further confirmation of both the early requirements for accessory cells in mixed lymphocyte reactions and the equivalence of accessory cells of responder or stimulator origin.

5.3.2 The tissue distribution of accessory cells

The distribution of accessory cells in various lymphoid populations is summarised in Figure 5.2. In addition to spleen cells, bone marrow cells peritoneal cells and, to a lesser extent, lymph node cells, all effected significant reconstitution of cytotoxic responses. Thymus cells, on the other hand, did not reconstitute the response. With the exception of peritoneal cells, optimal reconstitution was obtained with 2-4 x 10^6 cells. The drop in reconstituting capacity when more than 1 x 10^6 peritoneal cells were used probably reflects the toxic effects of the macrophages contained in this population. The reconstitution observed with lymph node cells suggests that this population contains accessory cells but these must be present in critical numbers before a response is observed.

The poor stimulator and reconstituting capacity of thymus cells indicates a paucity of accessory cells in this population. The finding that the response of lymph node cells to thymus stimulator cells can be augmented by the addition of irradiated responder spleen cells supports this suggestion (data not shown).

5.3.3 The capacity of adherent cells to reconstitute responses

As illustrated in Table 5.2, the addition of fresh, plastic adherent spleen cells of responder genotype to accessory cell deficient cultures resulted in complete reconstitution of the response while the addition of fresh, plastic adherent peritoneal cells of responder or stimulator

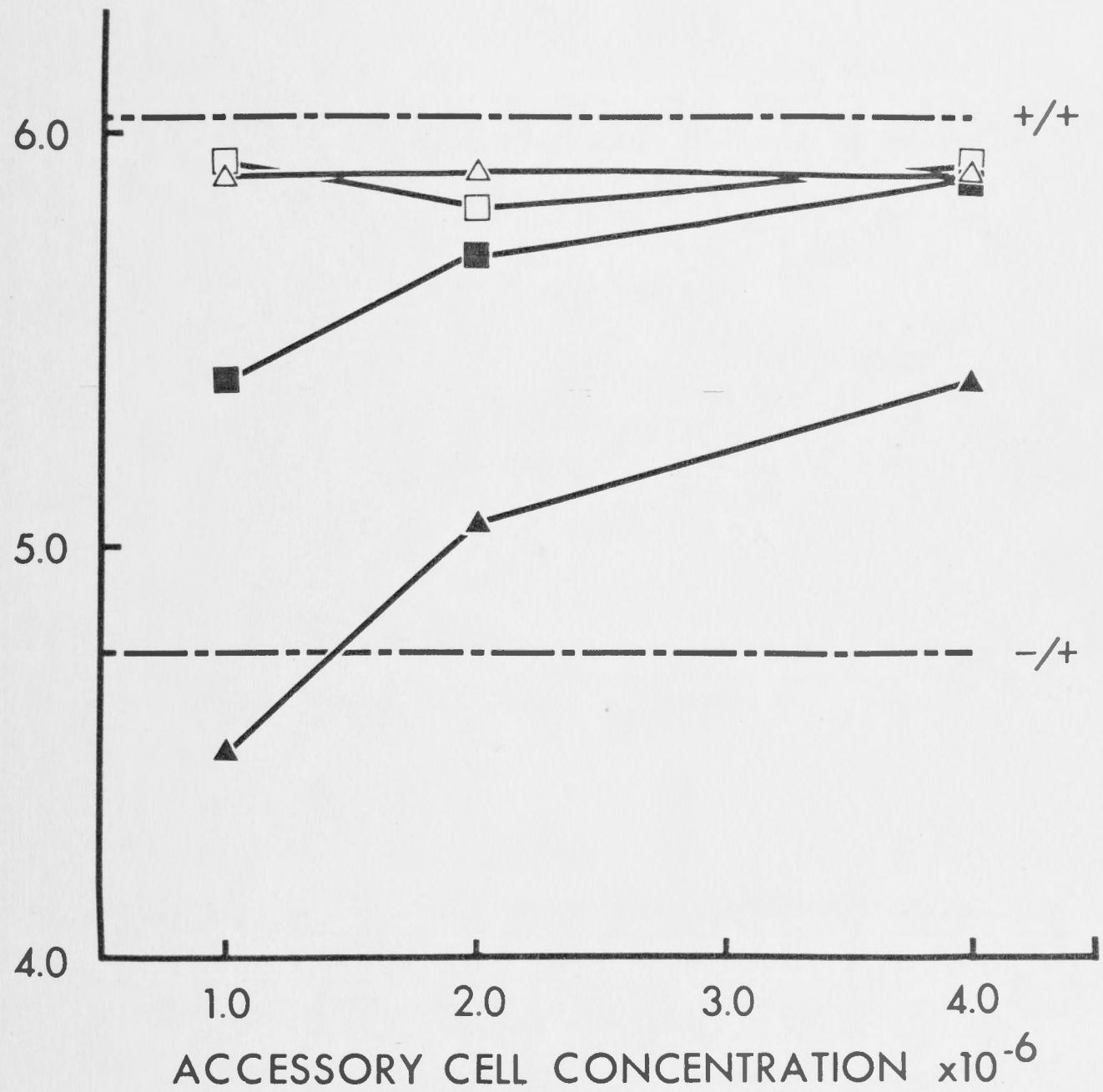

Figure 5.3 - Addition of Ig^+ or Ig^- irradiated responder spleens to adherent cell depleted cultures.

+/+, -/+ as for Figure 5.2.

Irradiated CBA/H spleen cell subpopulations added to (-/+) cultures.

■ , spleen cells; △ , Ig^+ cells; □ , $Ig^+ + Ig^-$ cells;

▲ , Ig^- cells.

TABLE 5.3

CHARACTERISATION OF THE ACCESSORY CELL POPULATION

Stimulator population[a]	Treatment of accessory cell population[b]	% cells removed by treatment	Cytotoxic activity log C.U./culture[g]
Carbonyl iron treated BALB/c spleen cells	Untreated	-	5.88
"	Anti-θ ascitic fluid + complement[c]	30%	5.72
"	Normal ascitic fluid + complement[d]	17%	5.91
"	Untreated CBA/H spleen cells mixed with SRBC then spun through Isopaque/Ficoll[e]	<5%	5.97
"	Untreated CBA/H spleen cells mixed with antibody coated SRBC then spun through Isopaque/Ficoll[f]	<5%	5.90
"	Untreated CBA/H spleen cells spun through Isopaque/Ficoll	<5%	6.02
"	No accessory cells	-	4.28
Untreated BALB/c spleen cells	"	-	5.74

Legends to Figure 5.3 -

a BALB/c irradiated stimulator cells were cultured with 2 x 10^6 CBA/H lymph node responder cells for 5 days then assayed on ^{51}Cr labeled P815 targets.

b Irradiated CBA/H spleen cells were used as a source of accessory cells throughout. Except where otherwise stated 4 x 10^6 cells were used.

c 2.8 x 10^6 cells added/culture.

d 3.3 x 10^6 cells added/culture.

e Untreated lymphocytes and SRBC were mixed in the proportions used for Ig rosetting, spun at 4°C, resuspended then spun through Isopaque/Ficoll.

f Same procedure as for (e), but untreated SRBC were substituted with SRBC coated with sheep anti-rabbit Ig.

g Background = 4.20 log C.U.

genotype partially reconstituted the response. In Chapter 4 it was shown that the incubation of the stimulator population on plastic surfaces did not remove all accessory cells. The corollary also applies in that non-adherent as well as adherent spleen cells were able to reconstitute the response (Table 5.2).

In contrast to freshly adherent cells, adherent peritoneal cells maintained in culture for three days prior to use did not reconstitute the response (Table 5.2). The inability of cultured macrophages to reconstitute the response was not the result of an increase in toxicity following culture since the incorporation of these cells into cultures of untreated stimulator and responder populations did not cause significant inhibition of the cytotoxic response at any of the cell concentrations tested (see Chapter 3, Table 3.4.B).

5.3.4 The reconstituting capacity of Ig^+ and Ig^- spleen cells

The reconstituting capacity of Ig^+ and Ig^- spleen cells is summarised in Figure 5.3. Over the dose range tested Ig^+ cells, remixes of Ig^+ and Ig^- cells and unfractionated spleen cells almost completely restored the response while Ig^- cells reconstituted poorly. The observation, in this experiment, that 1×10^6 cells from a remix of Ig^+ and Ig^- cells had a greater reconstituting potential than the same number of unfractionated spleen cells was not a consistent finding. It is unlikely that the accessory cells in the Ig^+ population are a subpopulation of contaminating T cells with passively acquired surface Ig since anti-θ serum and complement treated CBA/H spleen cells reconstituted the response as effectively as untreated populations (Table 5.3). The inferiority of the reconstitution observed with Ig^- cells suggests the presence of a limiting number of accessory cells in this population, possibly due to the incomplete separation of the Ig^+ and Ig^- subpopulations. Alternately a small subpopulation of accessory cells may be Ig^-. Carbonyl iron treatment of both populations completely removed their activity (data not shown). The possibility that

TABLE 5.4

RECONSTITUTION OF THE RESPONSE TO CARBONYL IRON TREATED STIMULATOR CELLS BY VARIOUS ACCESSORY CELL POPULATIONS

Stimulator population	Carbonyl iron treatment	Accessory cell population	Cytotoxic activity log C.U./culture[d]
4×10^6 BALB/c spleen cells	-[a]	-	5.90
"	+	-	4.48
"	+	4×10^6 Ia^- CBA/H spleen cells[b]	5.56
"	+	4×10^6 C' treated CBA/H spleen cells	5.87
"	+	1×10^5 L cells[c]	6.15
"	+	1×10^4 L cells	5.83

a + = treatment; - = no treatment.

b CBA/H spleen cells treated with anti-Ia serum + rabbit C'. (% cells lysed = 47%). Controls treated with rabbit C' alone (% cells lysed = 8%).

c L cells were mitomycin C treated.

d Cytotoxic activity assayed on ^{51}Cr labeled P815 cells. Background = 4.00 log C.U.

the accessory cells were Ig^- cells which rosetted naturally with SRBC was eliminated by carrying out the rosetting and separating procedures used for Ig separations with untreated CBA/H spleen cells and either untreated or sheep anti-rabbit Ig coated SRBC. The reconstituting activity of the cells remaining at the interface of the Isopaque/Ficoll was tested and found to be unimpaired in each case (Table 5.3).

5.3.5 The reconstituting capacity of Ia^- spleen cells

Ia^+ cells were removed from CBA/H spleen cell populations by treatment with anti-Ia serum and complement. The addition of Ia^- spleen cells to accessory cell depleted cultures resulted in a 10-fold increase in the response compared to a 20-fold increase with spleen cells treated with complement only (Table 5.4). Although the 2-fold difference is significant the data indicates that the majority of accessory cells are Ia^-.

It is also notable that mitomycin C treated L-929 cells were able to function as accessory cells. As shown in Table 5.4 the addition of 1×10^5 L cells to adherent cell depleted cultures completely restored the response. This data suggests that tumour cells, like L cells, supply both stimulating antigen and accessory cell requirements. The accessory cell requirement for stimulation with tumour cells was examined further with P815-X2 mastocytoma cells.

5.3.6 Accessory cell requirements for stimulation with neoplastic cells

As shown in this chapter and the previous one, lymph node cells used at 2×10^6/culture contain insufficient numbers of accessory cells to allow a response to accessory cell depleted lymphoid stimulator cells. The observation that P815 cells, L cells and IgA plasmacytoma cells, passaged *in vitro*, stimulate lymph node cells (Chapter 3, Table 3.1), suggests that the accessory cell requirements for induction of cytotoxic responses are less stringent with neoplastic cells than with lymphoid stimulator cells. Further investigations carried out with P815 cells showed that removal of

TABLE 5.5

ACCESSORY CELL REQUIREMENTS FOR STIMULATION WITH NEOPLASTIC CELLS

Carbonyl iron treatment of the stimulator[a]	Carbonyl iron treatment of responder[b]	Cytotoxic activity log C.U./culture[d]
-[c]	-	5.67
-	+	5.30
+	-	5.87
+	+	5.28

a Stimulator population 1×10^5 irradiated (5000 R) P815-X2 DBA/2 mastocytoma cells.

b Responder population, 2×10^6 CBA/H lymph node cells.

c - = no treatment; + = treatment.

d Cytotoxic activity estimated on labeled P815 target cells. Background = 4.03 log C.U.

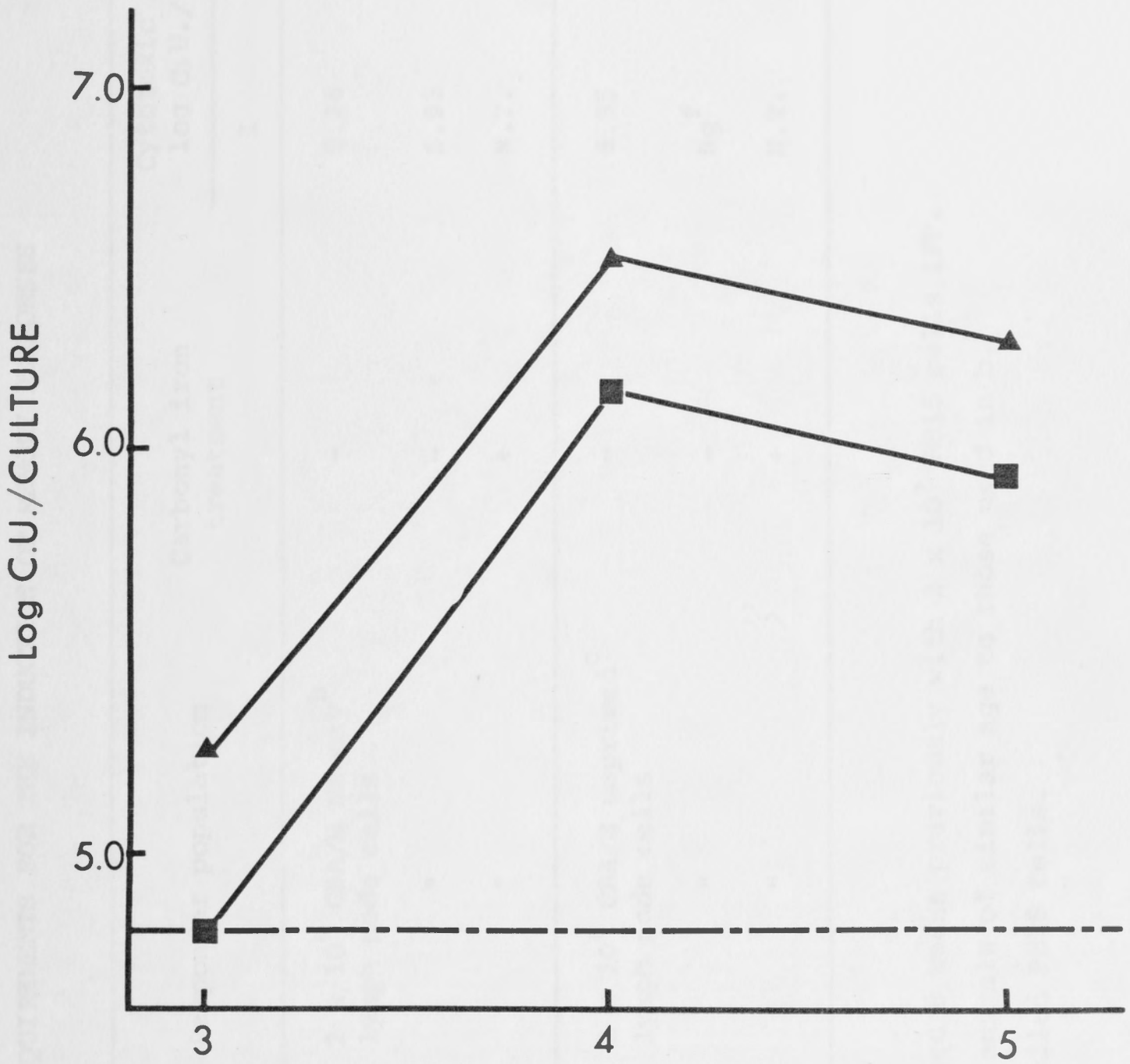

Figure 5.4 - The kinetics of memory responses.

Memory lymph node cells were obtained from CBA/H mice injected with 2×10^7 P815 cells intra footpad 8 weeks previously. Unprimed lymph node cells were obtained from untreated mice of similar age.

▲—▲, 4×10^6 irradiated BALB/c stimulator cells + 2×10^6 primed popliteal lymph node cells.

■—■, 4×10^6 irradiated BALB/c stimulator cells + 2×10^6 unprimed popliteal lymph node cells.

▬ ▬ ▬ = Background.

TABLE 5.6

THE ACCESSORY CELL REQUIREMENTS FOR THE INDUCTION OF MEMORY RESPONSES

Stimulator population	Carbonyl iron treatment	Responder population	Carbonyl iron treatment	Cytotoxic activity[a] log C.U./culture	
				I	II
4×10^6 BALB/c spleen cells	-[a]	2×10^6 CBA/H memory[b] lymph node cells	-	6.26	6.14
"	+	"	-	5.93	5.80
"	+	"	+	N.T.	N.T.
"	-	2×10^6 CBA/H unprimed[c] lymph node cells	-	5.95	6.13
"	+	"	-	Bg[f]	Bg[e]
"	+	"	+	N.T.	Bg

a -, no treatment; +, treatment.

b Popliteal lymph nodes from mice immunised 8 weeks previously with 2×10^7 P815 cells IFP.

c Popliteal lymph nodes from unimmunised animals of similar age to those used in b.

d Cytotoxic activity assayed on ^{51}Cr-labelled P815 cells.

e Bg = 3.98 log C.U.

f Bg = 4.77 log C.U. N.T. = not tested.

accessory cells from the cultures caused only a 2 - 2.5-fold decrease in the response (Table 5.5). This contrasted with the complete abrogation of the response when accessory cells were removed from cultures of lymphoid cells. Carbonyl iron treatment of the P815 cells had no significant effect on the response (Table 5.5).

In summary, stimulation with neoplastic cells, unlike stimulation with normal lymphocytes, appears to be relatively accessory cell independent.

5.3.7 The accessory cell requirements for the restimulation of primed responder cells

In a preliminary set of experiments the kinetics of the generation of cytotoxic T cells were established. Popliteal lymph nodes from mice injected intra-footpad 8 weeks previously with P815 cells were used as responder cells in mixed lymphocyte culture. Control cultures were set up with popliteal lymph node cells from unimmunised mice of similar age. As illustrated in Figure 5.4, the preimmunised cells showed the characteristics of memory populations, namely a premature and augmented cytotoxic response. Thus, in contrast to non-immune responder cells where no cytotoxic activity was detected before day 4 of culture, when memory cells were used as responders, significant cytotoxic activity was already in evidence by day 3 of culture. The peak cytotoxic response of memory cells was also significantly greater (2-fold) than that of unprimed mice. These results agree with those published by others (Röllinghoff and Wagner, 1975).

The requirement for accessory cells in secondary MLC was determined by culturing carbonyl iron treated stimulator cells with either unprimed or memory responder cells. Cultures were assayed for cytotoxic activity on days 3, 4 and 5 of culture but for simplicity only day 5 data has been tabulated. As shown in Table 5.6, when memory cells were used as responders, the removal of accessory cells from the stimulator population resulted in a 2-fold drop in cytotoxic activity while their removal from both stimulator and responder populations resulted in a 10-fold drop in activity.

In the latter case, however, the cytotoxic activity was still 10-fold greater than the background value. Conversely, when unprimed responder cells were used, removal of accessory cells from the stimulator population alone or from both the stimulator and responder populations, completely abrogated the response. Thus, the induction of secondary cytotoxic T cell responses appears to be less accessory cell dependent than the induction of primary responses.

D I S C U S S I O N

In the previous chapter it was shown that a population of cells present in stimulator spleen and depleted by treatment with carbonyl iron was required for the induction of cytotoxic T cells when lymph node cells were used as the responder population. Furthermore, carbonyl iron adherent cells removed from the stimulator population could be substituted with carbonyl iron adherent spleen cells of responder genotype. In this chapter a three-cell system was described which proved the equivalence of these two populations of accessory cells and facilitated their further characterisation.

The equivalence of accessory cells from responder and stimulator populations

Experiments using the three-cell system described, demonstrated that the accessory cells in the responder population resembled accessory cells of stimulator origin in the following ways:

(a) they were removed by carbonyl iron treatment,

(b) they functioned following irradiation,

(c) they were required in the first 24 hours of culture.

The effector cells generated were derived solely from the lymph node donor. A responder population comprised of irradiated spleen cells and syngeneic untreated lymph node cells therefore appeared to be equivalent to a responder population of untreated spleen cells. On the basis of these findings the three-cell system was used for the further characterisation of the accessory cells.

Cells behaving like accessory cells were found to be present in a number of lymphoid organs besides the spleen. They were also detected in significant numbers in lymph node, bone marrow and the peritoneal cavity but were not present in detectable amounts in thymus. The finding that lymph node cells reconstituted the response suggests that critical numbers of accessory cells must be present before a response is induced. The suppressive effects normally observed following the use of more than 2×10^6 lymph node responder cells/culture (see Chapter 2, Figure 4.4) seemed to be overcome when the extra lymph node cells were irradiated. The deficiency of accessory cells in thymus populations has also been reported by others (Dyminski and Smith, 1975, 1976) and probably accounts for the poor stimulation observed with this population when lymph node responder cells are used. Preliminary experiments have shown that the response to alloantigens presented on thymus cells can be considerably enhanced when combinations of spleen and lymph node responder cells are used.

The fact that accessory cells from any source function following irradiation suggests that the cells involved are either radio-resistant or active very early in the response. If the first alternative applies the radio-resistant population is not included in the radio-resistant, long-lived, adherent macrophage subpopulation since these are unable to function as accessory cells. However, the demonstration that accessory cells are required early in the response lends considerable support to the second alternative.

Adherence properties of accessory cells

Data described in Chapter 3, which showed that both plastic adherent and non-adherent cells were equally capable of stimulating strong cytotoxic T cell responses, inferred that both of these populations contained adequate numbers of accessory cells. In this chapter data was presented which confirmed that plastic adherent and non-adherent spleen cells (and peritoneal cells) are indeed a rich source of accessory cells. The fact that considerable numbers of accessory cells are not plastic adherent also explains why preincubation of both the responder and the stimulator populations on plastic prior to culture had no significant effect on the subsequent cytotoxic T cell response (see Chapter 4). Since mature macrophages were unable to function as accessory cells it seems unlikely that the plastic adherent accessory cells belong to this subpopulation. Accessory cells from the spleen and peritoneal cavity therefore appear to fall into at least two subsets - a plastic adherent, and a non-plastic adherent - both of which are removed by carbonyl iron but neither of which includes the mature macrophage. Whether the accessory cells are all members of the same cell class expressing varying degrees of "stickiness" remains to be determined. These conclusions deviate somewhat from those reached by others in studies of the adherent cell requirements in MLC. For instance, Wagner *et al.* (1972), concluded that the cell population removed by glass bead columns which was required for optimal proliferative and cytotoxic T cell responses was a macrophage since it was also removed by anti-macrophage serum and complement treatment and, further, could be replaced by T cell depleted peritoneal exudate cells, a population considerably enriched for macrophages. However, since the whole T cell depleted peritoneal exudate cell population and not the adherent, long-lived, subpopulation was used as a source of accessory cells, the possibility was not excluded that the active reconstituting moieties were B cells or immature cells which were also targets for the anti-macrophage serum. Likewise,

MacDonald *et al.* (1973b) in studies involving the removal of accessory cells by incubation on plastic surfaces or their inactivation by treatment with actinomycin D, also concluded that macrophages were the active cells. This conclusion could be erroneous however, since cells other than macrophages are known to adhere to plastic and accessory cells other than macrophages may be affected by actinomycin D. The former criticism can also be levelled at the early studies on the adherent cell requirements for proliferation in human MLC where unfractionated or freshly adherent cells were used as a source of accessory cells (Rode and Gordon, 1970; Twomey *et al.*, 1970). In a more recent study, however, Rode and Gordon (1974) showed that long-lived adherent cells were able to reconstitute the proliferative response in cultures of adherent cell depleted human lymphocytes. The difference between these results and those obtained in this study suggest that human macrophages and mouse macrophages may differ in their accessory cell capacity.

Surface markers on accessory cells

Cells with accessory cell activity were found to separate predominantly with the Ig^+ subpopulation of spleen cells. Control studies involving anti-θ and C' treatment of the CBA/H spleen cell population and rosetting with antibody treated or untreated SRBC indicated that the accessory cell was neither a T cell nor any other Ig^- cell passively separating with the Ig^+ cells. The weak reconstituting and stimulator activity of Ig^- cells suggests that small numbers of accessory cells do remain in this fraction. Since anti-θ and C' treatment does not remove these (data not shown) they are possibly immature cells or cells with only a small amount of surface Ig.

The stimulation observed with FcR^- and CR^- spleen populations in cultures containing limiting numbers of responder accessory cells (see Chapter 3) suggests that all accessory cells do not bear these two receptors. Accordingly, it is unlikely that the Ig^+ accessory cells all

exhibit surface Ig as a result of binding exogenous Ig via Fc receptors. The active population may therefore represent a subpopulation of adherent B cells or alternatively may be comprised of a combination of Fc receptor bearing immature macrophages and B cells. Treatment of lymphocytes, firstly with trypsin to remove any immunoglobulin bound passively via Fc receptors and then, after a recovery period, with anti-mouse Ig μ chains or IgG Fab fragments which would not bind to regenerated Fc receptors, may help to clarify this point.

Since the removal of Ia^+ cells from either the accessory cell or the stimulator cell populations had little effect on the response (Chapter 3 and this chapter) it is unlikely that the majority of accessory cells bear this surface marker. Again this implies that if the accessory cells are B cells they must represent a relatively immature subpopulation since the majority of mature B cells exhibit surface Ia antigens (for review see Hämmerling, 1976).

To summarise, the accessory cell population required for the induction of cytotoxic T cells *in vitro* appears to be comprised predominantly of Ig^+, θ^-, $FcR^\pm$, $CR^\pm$, Ia^- cells which are not mature macrophages. It should be stressed, however, that the inability to detect complement and Fc receptors or Ia antigens on the surface of accessory cells may be a reflection of the sensitivity of the methods employed. These cells may thus have small, but so far undetected amounts of any one, or all, of these surface markers. It is notable that mitomycin C treated L cells are also able to function as accessory cells. This result implies that accessory cell activity is not restricted to lymphoid cells alone.

A splenic accessory cell population resembling the one described here has also recently been described by Dyminski and Smith (1975, 1976). They showed that the induction of both proliferative and cytotoxic T cell responses in cultures of allogeneic thymus cells was totally dependent on the presence of Ig^+, CR^+, θ^- cells which were not mature macrophages.

Furthermore, this population could be syngeneic with either the responder or the stimulator population and was required at the initiation of the response.

Two other studies exist where the inclusion of splenic accessory cells in mixed lymphocyte cultures induced enhanced cytotoxic responses. In one study by Hodes *et al.* (1974), the synergising accessory cell was characterised as a nylon wool adherent, θ^-, non-macrophage. In the other study two populations of synergising accessory cells were described in nude spleens. The first of these was radio sensitive, was not retained on glass bead columns and had to be allogeneic with the stimulator before it could synergise (Schilling *et al.*, 1976). The second was radio resistant, adherent and functioned whether syngeneic or allogeneic with the stimulator population (Miller *et al.*, 1976). It was suggested that the first population gave rise to the second following stimulation with alloantigen. Whether the second population was a macrophage or a cell resembling the one described here was not determined.

So far, the latter study is the only one reported where accessory cells resembling those described here have had to recognise stimulator cell antigens in order to function. Recognition of stimulator (or responder) alloantigens by the accessory cells described in this study is an unlikely requirement since responder accessory cells function as well as stimulator accessory cells when combinations of F_1 stimulator and parental responder cells are used (data not shown). Furthermore, as will be shown in the two following chapters, immunoincompetent foetal lymphoid cells and supernatants from unstimulated spleen cell cultures are both capable of fulfilling accessory cell functions.

Accessory cell requirements for responses to neoplastic stimulator cells and for memory responses

The response of unprimed T cells to neoplastic cells was found to be significantly less dependent on the presence of accessory cells than the

response to resting lymphocytes. Any explanation of this phenomenon requires some prior indication of the role that accessory cells may play in mixed lymphocyte reactions. In the formulation of any theory on the requirements for the induction of primary responses the prime consideration must be that recognition of antigen alone is insufficient for the induction of cytotoxic T cells. Suggestions for the role that accessory cells fulfil in the induction of T cell responses will be considered in greater detail in Chapter 8, but briefly, two possibilities are that accessory cells:

(a) produce signals required, in addition to antigen, which activate cytotoxic T cell precursors directly,

(b) activate lymphocytes so that they in turn transmit such inductive signals.

Neoplastic cells may circumvent the critical requirement for accessory cells observed with resting lymphocytes because they are already "activated" or because they have the properties of both stimulator and accessory cells. The observation that L cells can function as accessory cells provides some evidence for the latter alternative.

It is now well established that the requirements for the restimulation of pre-primed allo-reactive T cells are much less stringent than those required for primary responses. Thus, UV and heat treated cells and sub-cellular fragments all induce secondary but not primary responses (Engers *et al.*, 1975; Röllinghoff and Wagner, 1975; Wagner *et al.*, 1976; Häyry and Andersson, 1976). The question then arises as to whether this difference represents a change in the receptor specificity or density on the memory T cell or alternatively reflects an independence of inductive stimuli (or second signal) not supplied by antigen alone. At this stage there is no answer to this question but if accessory cells do, in fact, either directly or indirectly result in the production of inductive stimuli memory T cells may be less dependent on this signal than unprimed cells.

S U M M A R Y

In this chapter a three-cell system comprising irradiated BALB/c and CBA/H spleen cells and untreated CBA/H lymph node responder cells was described and subsequently used to characterise the accessory cell population required for the generation of cytotoxic T cells *in vitro*. Accessory cells of stimulator and responder origin were shown to be equivalent populations since they were both depleted by treatment with carbonyl iron, functioned following irradiation and were required in the first 24 hours of the response. In addition to the spleen, accessory cells were also found in lymph node, bone marrow and the peritoneal cavity but were absent from the thymus. The active population in the spleen was shown to be predominantly Ig^{+}, θ^{-}, $CR^{\pm}$, $FcR^{\pm}$, and Ia^{-} and did not include mature macrophages. The lack of mature B cell markers on these cells suggested that they may be immature B cells although the possibility of their being immature macrophages was not eliminated. Accessory cells could be further divided into plastic adherent and plastic non-adherent subpopulations suggesting that they exhibited varying degrees of "stickiness".

In contrast to primary responses, secondary responses were shown to be considerably less dependent on the presence of accessory cells. Similarly, when neoplastic cells were used as a source of stimulators rather than resting lymphocytes, the response was markedly less accessory cell dependent. Possible explanations for these differences in accessory cell requirements were discussed.

CHAPTER 6

THE ONTOGENY OF STIMULATOR AND ACCESSORY CELL FUNCTION

6.1 Introduction

In studies on the ontogeny of stimulator activity several investigators have shown that the strength of the proliferative response generated in cultures of immature stimulator cells and adult allogeneic responder cells increased with the age of the donor animal until adult levels were reached, generally well after birth. This was shown to be true for embryonic chicken spleen (Lafferty *et al.*, 1972), foetal mouse spleen and liver (Mosier, 1974), foetal rat skin (Lane *et al.*, 1975) and neonatal mouse spleen and thymus (Howe and Manziello, 1972; Adler *et al.*, 1970; Wagner and Wyss, 1973). In no instance was significant stimulator activity detected before day 18 of gestation. From their studies with embryonic chicken spleen cells Lafferty *et al.*, (1972), concluded that lymphoid cells in addition to being metabolically active must also be immunocompetent before they can function as stimulator cells.

In contrast to the proliferative response only one study exists on the ontogeny of cells capable of inducing cytotoxic T cell responses and this involved the use of neonatal rather than foetal tissue (Wagner and Wyss, 1973). Nonetheless the findings were of considerable interest since they suggested that the stimulation of optimal cytotoxic T cell responses, unlike optimal proliferative responses, was not related to the age of the stimulator cell donor. Allogeneic 14 day foetal mouse liver cells have also been shown to immunise mice against a subsequent challenge with skin grafts syngeneic with the immunising population suggesting that foetal as well as neonatal cells may be capable of inducing cytotoxic T cell responses (Möller, 1963).

In addition to the ontogeny of the stimulator cell the ontogeny of the accessory cell is also of interest since recent reports involving

studies of the *in vitro* induction of antibody responses have suggested that cell activity, like the capacity to induce proliferative responses in MLC, may be related to the age of the cell donor (Hirsch *et al.*, 1970; Hardy *et al.*, 1973; Landahl, 1976).

The following investigations were undertaken to determine when, if ever, foetal liver populations contain cells fulfilling either a stimulator or an accessory role in the induction of cytotoxic T cells *in vitro*.

6.2 Methods and materials

6.2.1 Animals

CBA/H and BALB/c mice were used in all experiments. Two to three month old females were housed with males and examined daily for vaginal plugs. The appearance of a plug was taken as day zero, and birth occurred on day 19.

6.2.2 Preparation of cell suspensions

Foetal liver cells were removed and suspensions made in F-15 containing 10% FCS by gently pressing the organs through a fine stainless steel grid. Cells were pooled from 2-3 litters of the same gestational age. The cells were then washed three times in medium by centrifuging at 400 g for 5' then declumped once more. Viability was estimated by trypan blue exclusion. Spleen and lymph node cell preparations were made as described in Chapter 2.

6.2.3 ^{51}Cr-labelled targets

^{51}Cr-labelled P815-X2 mastocytoma cells (P815), L-929 cells (L cells) and macrophages were prepared as described in Chapters 2 and 3 (Sections 2.2.5 and 3.2.2).

6.2.4 Treatment of cells with anti-θ and complement

Anti-θ ascitic fluid was prepared and used as described in Chapter 3 (Sections 3.2.5 and 3.2.6 respectively).

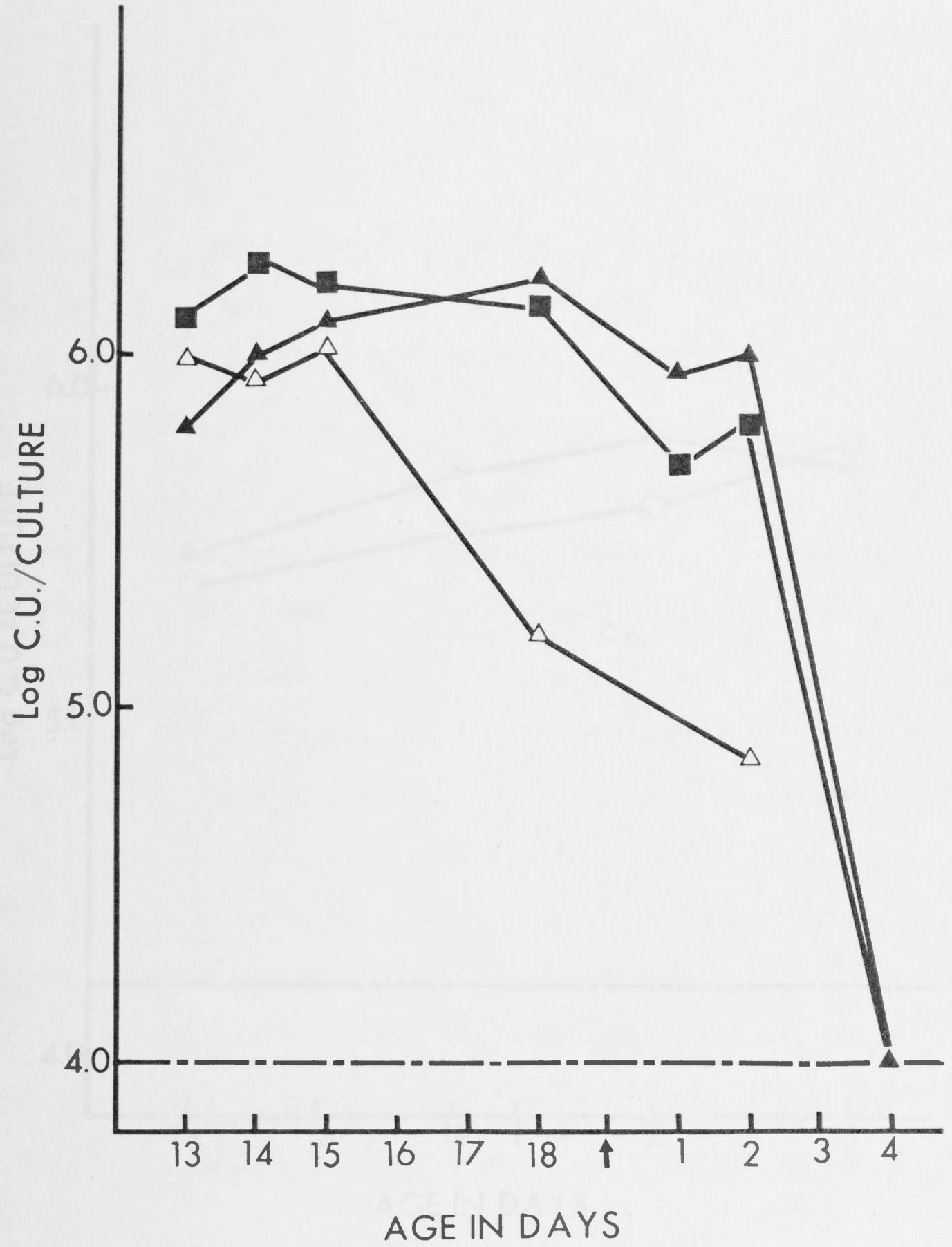

Figure 6.1 - The ontogeny of BALB/c foetal liver stimulator cells.
△—△, 4 x 10^6 stimulator cells + 4 x 10^6 spleen responder cells.
■—■, 2 x 10^6 stimulator cells + 4 x 10^6 spleen responder cells.
▲—▲, 2 x 10^6 stimulator cells + 2 x 10^6 lymph node responder cells.
Irradiated BALB/c foetal liver cells were used as stimulators and CBA/H cells as responders throughout. † denotes birth. Figures to the left ot the arrow are days of gestation, and to the right, days after birth.
Background = 4.00 log C.U.

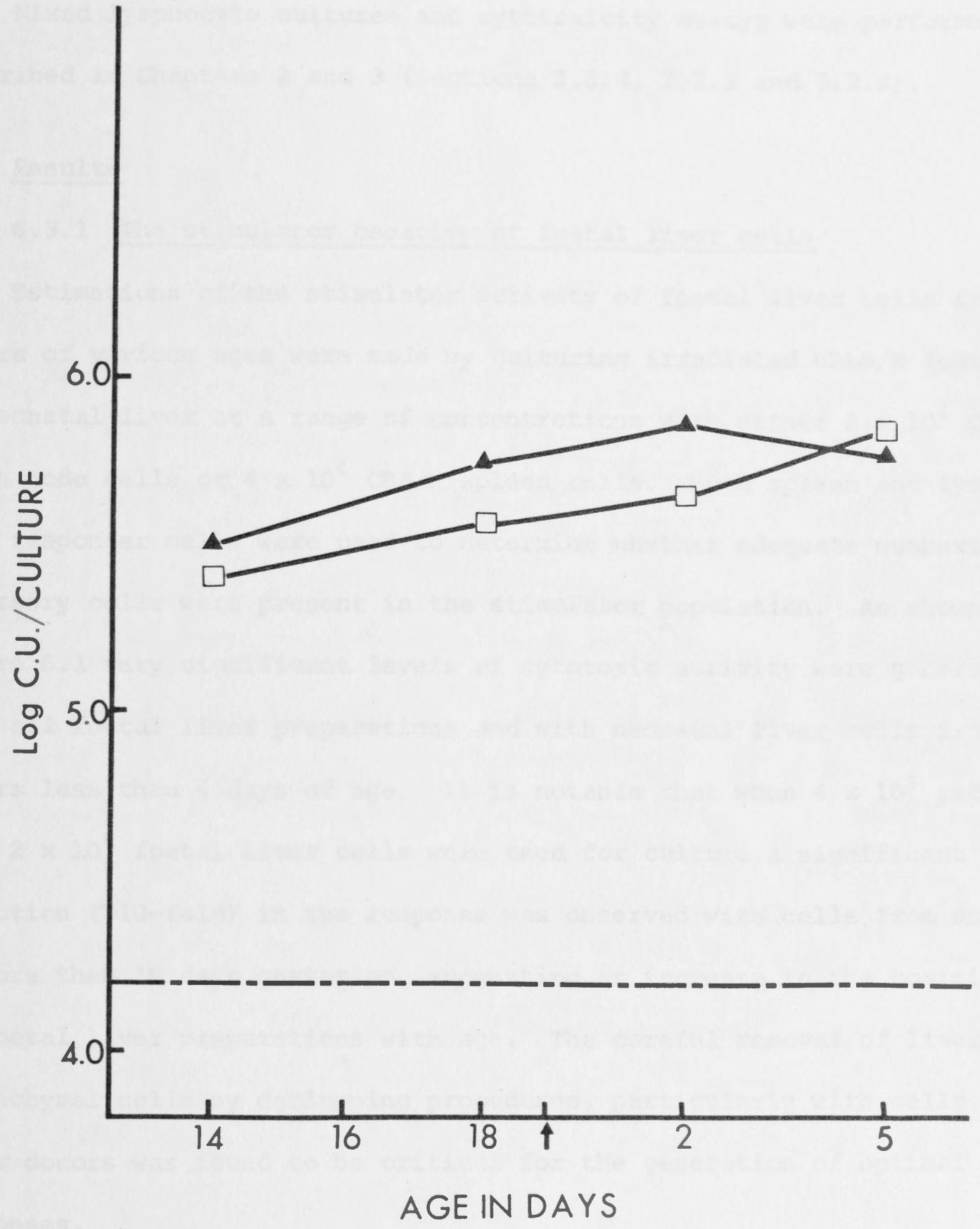

Figure 6.2 - The ontogeny of CBA/H foetal liver stimulator cells.
▲—▲, 2 x 10^6 stimulator cells + 2 x 10^6 lymph node responder cells.
□—□, 2 x 10^6 stimulator cells + 4 x 10^6 spleen responder cells.
Irradiated CBA/H foetal liver cells were used as stimulator cells and BALB/c cells as responders throughout. ↑ denotes birth. Figures to the left of the arrow are days of gestation, and to the right, are days after brith.
Background = 4.20 log C.U.

6.2.5 Tissue culture procedures

Mixed lymphocyte cultures and cytotoxicity assays were performed as described in Chapters 2 and 3 (Sections 2.2.4, 2.2.5 and 3.2.2).

6.3 Results

6.3.1 The stimulator capacity of foetal liver cells

Estimations of the stimulator activity of foetal liver cells from donors of various ages were made by culturing irradiated BALB/c foetal or neonatal liver at a range of concentrations with either 2×10^6 CBA/H lymph node cells or 4×10^6 CBA/H spleen cells. Both spleen and lymph node responder cells were used to determine whether adequate numbers of accessory cells were present in the stimulator population. As shown in Figure 6.1 very significant levels of cytotoxic activity were generated with all foetal liver preparations and with neonatal liver cells from donors less than 4 days of age. It is notable that when 4×10^6 rather than 2×10^6 foetal liver cells were used for culture a significant reduction ($\sim$10-fold) in the response was observed with cells from donors of more then 16 days gestation, suggesting an increase in the toxicity of foetal liver preparations with age. The careful removal of liver parenchymal cells by declumping procedures, particularly with cells from older donors was found to be critical for the generation of optimal responses.

The observation that very similar levels of cytotoxic activity were generated with either spleen or lymph node responder populations indicated that accessory cells were not limiting in foetal liver at any of the gestation periods tested.

Irradiated CBA/H foetal and neonatal liver cells were also able to stimulate significant cytotoxic responses when cultured with BALB/c lymph node or spleen responder cells (Figure 6.2). However, in contrast to BALB/c, CBA/H neonatal liver cells were still strongly stimulatory 5 days

TABLE 6.1

SPECIFICITY OF CYTOTOXIC CELLS INDUCED BY ALLOGENEIC FOETAL AND NEONATAL LIVER CELLS

Stimulator population	Gestation period (days)	log C.U./culture			
		P815 cell targets[e]	BALB/c macrophage targets[d]	L cell targets	CBA/H macrophage targets
A. 2×10^6 BALB/c foetal liver cells[a]	13	5.80	6.17	Bg[c]	N.T.
"	15	5.99	6.06	"	"
"	16	6.10	5.91	"	"
"	18	6.23	6.06	"	"
"	2 day neonatal	6.01	6.10	"	"
"	4 day neonatal	Bg	N.T.	N.T.	"
2×10^6 BALB/c adult liver[a]	-	Bg	N.T.	N.T.	N.T.
4×10^6 BALB/c adult spleen[a]	-	6.05	5.98	Bg	N.T.
B. 2×10^6 CBA/H foetal liver cells[b]	16	Bg	N.T.	5.72	5.79
4×10^6 CBA/H adult spleen[b]	-	Bg	N.T.	5.63	5.74

a 2×10^6 CBA/H lymph node responder cells used throughout. All stimulator cells were irradiated (1000 R).

b 2×10^6 BALB/c lymph node responder cells used throughout.

c Background values for all targets were <4.4 log C.U.

d Assays on either ^{51}Cr labeled BALB/c or CBA/H macrophages were of 14 hrs duration.

e Assays on ^{51}Cr labeled P815 or L cell targets were of 4 hrs duration. N.T. = not tested; Bg = Background.

after birth. These observations possibly reflect a difference between the two strains in the rate with which their livers differentiate into adult form since neonatal BALB/c livers always macroscopically resembled adult livers sooner after birth than did CBA/H livers.

6.3.2 The nature of the effector population

In the previous section it was demonstrated that allogeneic foetal liver cells from as early as day 13 of gestation have the capacity to induce cytotoxic responses in MLC. In this section the identity of the effector population and its specificity are established.

As illustrated in Table 6.1.A and B the cytotoxic cells induced by foetal liver stimulator cells from donors of all ages tested were specific for the sensitising antigens. Thus CBA/H lymph node cells stimulated with BALB/c foetal liver cells lysed P815 ($\underline{H\text{-}2}^{d}$) and BALB/c macrophage targets but not L cell ($\underline{H\text{-}2}^{k}$) targets. Conversely BALB/c lymph node cells sensitised with CBA/H foetal liver cells lysed L cell and CBA/H macrophage targets but not P815 targets. The fact that both macrophage and neoplastic cell targets were specifically lysed suggested that the sensitising antigens were predominantly H-2 antigens and not oncofoetal antigens. It is also notable that the cytotoxic activity induced by both foetal liver cells and adult spleen cells was of similar magnitude.

The activity of both BALB/c (Table 6.2) and CBA/H effector populations was completely abrogated following treatment with anti-θ ascitic fluid and complement, strongly suggesting that the active population was comprised solely of cytotoxic T cells.

In summary, allogeneic foetal liver stimulator cells induced cytotoxic T cell responses of similar magnitude and specificity to those induced by allogeneic adult lymphoid organs.

TABLE 6.2

REMOVAL OF EFFECTOR CELL ACTIVITY WITH ANTI-θ ASCITIC FLUID AND COMPLEMENT

Treatment of effector population[a]	Log C.U./culture[b]
Untreated	5.73
Anti-θ ascitic fluid + C'	4.38
Normal ascitic fluid + C'	5.57

a Effector cells from a culture of 2×10^6 irradiated BALB/c 16-day foetal liver cells and 2×10^6 CBA/H lymph node responder cells assayed on day 5.

b Cultures were assayed on 1×10^5 ^{51}Cr labeled P815 targets.

Background = 4.31 log C.U.

6.3.3 The accessory cell content of foetal liver preparations

There are two possible explanations for the independence of responder accessory cells when foetal liver cells are used as a source of stimulator cells:

(a) Stimulation by foetal cells (like neoplastic cells) is relatively independent of accessory cells.

(b) Foetal liver populations contain significant numbers of accessory cells.

(a) Carbonyl iron treatment of foetal liver cells - To test the first alternative liver cells from 16 day CBA/H foetuses were treated with carbonyl iron prior to culturing with BALB/c lymph node cells. This treatment removed approximately 30% of the foetal liver cells and resulted in complete abrogation of the response (Table 6.3). Furthermore, the response to carbonyl iron treated stimulator cells could not be restored by either the addition of irradiated responder spleen cells to the cultures or by the use of spleen rather than lymph node responder cells (Table 6.3). Similar results were also obtained with cells from 14, 17 and 19 day CBA/H foetuses.

These results contrasted with those obtained with adult lymphoid populations (see Chapter 5) and suggested that in foetal liver either two populations, one with stimulator activity and another with accessory cell activity or possibly one population exhibiting both properties were removed by treatment with carbonyl iron. The question of the degree of accessory cell dependence in this culture system thus remained unresolved.

(b) Three cell experiments with foetal liver accessory cells - To determine whether a subpopulation of cells existed in foetal liver with properties resembling those of the accessory cells found in adult lymphoid organs, irradiated CBA/H foetal liver cells from 14, 16, 17 and 19 day donors were cultured with untreated or carbonyl iron treated,

TABLE 6.3

THE EFFECTS OF CARBONYL IRON TREATMENT

OF FOETAL LIVER STIMULATOR CELLS

Stimulator population	Carbonyl iron treatment	Responder population	Cytotoxic activity log C.U./culture
3×10^6 liver cells from 16-day CBA/H foetuses	-[c]	2×10^6 BALB/c lymph node cells	5.72[a]
"	+	"	Bg[b]
"	-	4×10^6 BALB/c spleen cells	5.93
"	+	"	4.49
"	-	2×10^6 BALB/c lymph node cells + 4×10^6 BALB/c irradiated spleen cells	5.68
"	+	"	Bg

a Cytotoxic activity assayed on 2×10^4 ^{51}Cr labeled L-929 targets.

b Bg = 4.33 log C.U.

c Carbonyl iron treatment removed 30% of the foetal liver population.

+ = treatment; - = no treatment.

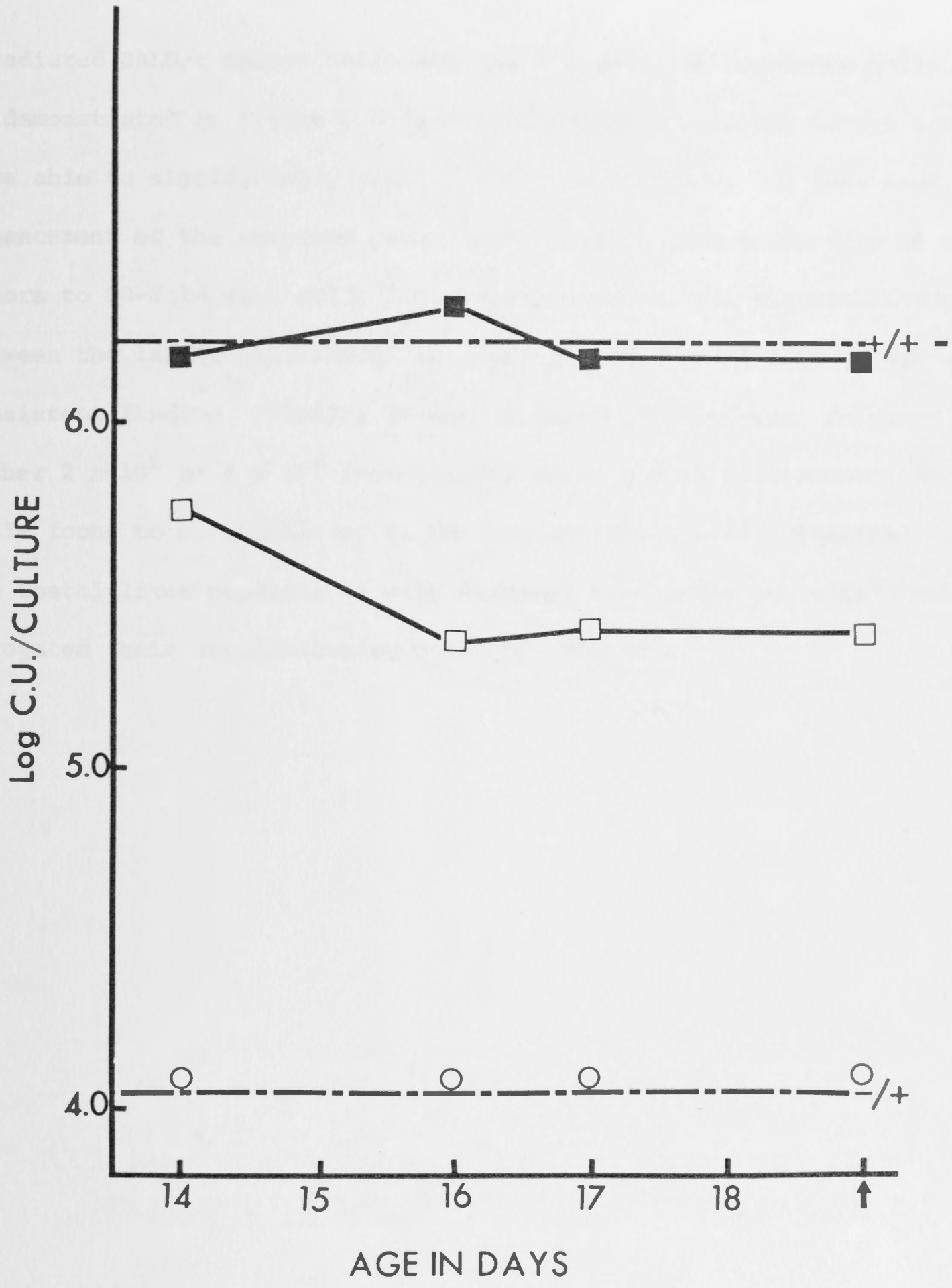

Figure 6.3 - The accessory cell activity of CBA/H foetal liver cells.
+/+ line denotes cultures comprised of 4 x 10^6 irradiated BALB/c spleen stimulator cells and 2 x 10^6 CBA/H lymph node responder cells.
-/+ line same as +/+ but stimulator cells were treated with carbonyl iron prior to irradiation. To determine the accessory cell contant of CBA/H foetal liver cells 4 x 10^6 irradiated cells from 14, 16, 17 and 19 day donors were added to -/+ cultures (□). As a control the same cells were added to +/+ cultures (■).
○, 4 x 10^6 irradiated, carbonyl iron treated foetal liver cells were added to -/+ cultures. ↑ denotes birth. Figures to the left of the arrow are days of gestation.

irradiated BALB/c spleen cells and CBA/H lymph node responder cells. As demonstrated in Figure 6.3 foetal liver cells from all donors tested were able to significantly reconstitute the response. In this experiment enhancement of the response ranged from 22-fold with cells from 19 day donors to 50-fold with cells from 14 day donors. The apparent difference between the latter populations in their accessory cell content was not a consistent finding. Similar levels of reconstitution were observed with either 2×10^6 or 4×10^6 foetal liver cells and in no instance were the cells found to be inhibitory at the concentration used. Treatment of the foetal liver populations with carbonyl iron prior to culture completely abrogated their reconstituting activity (Figure 6.3).

DISCUSSION

A. Foetal liver stimulator cells - It was established in this chapter that from as early as day 13 of gestation allogeneic foetal liver cells were able to induce cytotoxic T cell responses of similar magnitude and specificity to those induced by adult lymphoid populations. The stimulator activity of foetal liver cells was completely removed by carbonyl iron treatment but unlike adult lymphoid cells this loss in activity did not appear to be the result of selective depletion of non-stimulating accessory cells since the replacement of these did not restore the response. All stimulator cells in foetal liver from day 13 of gestation onwards therefore appeared to be adherent or "sticky" cells. A number of cell types known to be present in foetal liver at this time fit this description.

Thus, from day 13 of gestation foetal liver contains varying numbers of non-haemopoietic cells, pluripotent stem cells, granulocytic cells, mature and immature macrophages (Cline and Moore, 1972) and Ig^+ cells Melchers *et al.*, 1975; Melchers and Phillips, 1976; Rosenberg and Parish, 1976).

Using a rosetting technique and the same CBA/H strain mice used in this study Rosenberg and Parish (1976) described a subpopulation of cells in foetal liver with low levels of surface Ig which were first detectable on day 13 of gestation when they represented approximately 2% of the population. Their numbers increased steadily to about 15% by day 18 of gestation and thereafter declined rapidly. These cells, which did not appear to acquire their surface Ig passively and were therefore thought to be immature B cells, were completely removed by treatment with carbonyl iron but not by incubation on plastic surfaces. After birth the majority of Ig^+ cells resembled mature B cells and were not removed by treatment with carbonyl iron. Melchers *et al.* (1975), and Melchers and Phillips

(1976) have also described a similar population of immature B cells in foetal liver. Unfortunately the adherence properties of these cells were not studied.

The carbonyl iron adherent stimulator cells in foetal liver may therefore include any one or all of the following: mature or immature macrophages, immature B cells and perhaps other haemopoietic cells such as normoblasts which make up a considerable portion of the foetal liver population at all stages of its development (Cline and Moore, 1972). The finding that 30% - 40% of the foetal liver cells are removed by carbonyl iron from day 14 of gestation onwards certainly suggests that cells other than macrophages and B cells are removed by this treatment since the latter populations combined only make up approximately 5% of the population on day 13-14 of gestation and approximately 20% by day 18 of gestation (Cline and Moore, 1972; Rosenberg and Parish, 1976). The fact that only small numbers of B cells and macrophages are present on day 13-14 of gestation when optimal stimulation was already in evidence does not preclude their being the only cells with stimulator activity since similar numbers of neoplastic cells induce very significant cytotoxic activity (see Chapter 3).

The stimulation observed with neonatal liver cells is probably caused by more mature lymphoid cells or macrophage-like cells since the haemopoietic activity of the liver declines rapidly soon after birth (Metcalf and Moore, 1971; Stutman *et al.*, 1970). It will be interesting to see whether neonatal stimulator cells are also removed by carbonyl iron.

The decline in haemopoietic activity in liver after birth combined with the migration of cells from the liver to the bone marrow and spleen probably also explains the early and rapid loss of stimulator activity after birth observed with BALB/c neonatal liver cells. An increase in the number of liver parenchymal cells, which in adult liver have been found to be highly toxic in culture (personal observation), probably also

contributes to the decline in the response. The fact that day 5 CBA/H neonatal liver cells still stimulated strong cytotoxic T cell responses suggests that haemopoietic activity may be sustained for longer in this population than in the BALB/c population. The depression of the response which occurred when the number of BALB/c foetal liver cells from donors of more than 16 days gestation was increased from 2×10^6 to 4×10^6/ culture may reflect the presence of immunosuppressive agents in foetal liver which must be present in critical concentrations to be effective. Two possible sources of suppression are granulocytic cells which are known to be toxic in culture (Rode and Gordon, 1974) and whose concentration increases quite dramatically about the time suppression is first observed (Cline and Moore, 1972) and $alpha_1$-foetoprotein a product of foetal cells which has been reported to inhibit MLC responses (Murgita and Tomasi, 1975).

In a syngeneic MLC system involving combinations of foetal liver stimulator cells and adult spleen responder cells Chism *et al.* (1976), also noted an immunosuppressive effect when stimulator:responder ratios were increased above a certain critical level.

The existence of immunosuppressive cells and factors plus the fact that many early studies were performed in the absence of 2-ME and at only one stimulator and responder cell concentration may explain the failure of others to detect stimulator cells in early foetal liver preparations. The possibility cannot be excluded, however, that the cells in foetal liver which are capable of inducing optimal cytotoxic responses may be incapable of inducing optimal proliferative responses. Evidence in support of this suggestion comes from studies by Wagner and Wyss (1973), who observed that neonatal spleen cells induced optimal cytotoxic T cell responses from birth onwards whereas optimal proliferative responses were not induced until the donors were well over 10 days of age. The recent

demonstration by Press (1975), that adult levels of Ia^+ cells in spleen are not reached until well after 16 days of age may perhaps explain this difference.

Recently it has been shown that syngeneic foetal liver cells, from as early as day 14 of gestation, can stimulate cytotoxic T cell responses specific for oncofoetal antigens (OFA). Since OFA are found on a variety of foetal and neoplastic cells but not on the equivalent normal adult cell type the demonstration in the present study that effector T cells raised against allogeneic foetal liver cells lysed adult macrophage targets as efficiently as neoplastic cell targets suggested that the sensitising antigens in the allogeneic system were predominantly H-2 antigens and not OFA.

B. Foetal liver accessory cells - The general lack of surface markers apart from Ig on the accessory cell population found in adult spleen implied that these cells were possibly represented by an immature subpopulation of B cells.

In this chapter data was presented which convincingly demonstrated that immature, immunoincompetent cells in the form of foetal liver cells were quite capable of performing an accessory cell function. Furthermore, these cells, like those in adult lymphoid organs were also removed by carbonyl iron. The fact that these cells are immunoincompetent provided further evidence that the co-operation observed with accessory cells in MLC does not require the specific recognition of either the stimulator or the responder population.

The carbonyl iron adherent accessory cell population in adult spleen is predominantly Ig^+, θ^-, $FcR^{\pm}$, $CR^{\pm}$, and Ia^-. A similar carbonyl iron adherent subpopulation of cells which was Ig^+, θ^-, CR^-, $FcR^{\pm}$ has been demonstrated in foetal liver from day 13 of gestation (Rosenberg and Parish, 1976). Whether Ia antigens are also exhibited on this population

or on any other lymphoid or macrophage-like population in foetal liver is unresolved. Although Ia antigens have been isolated and characterised in 14-15 day foetal liver cells the nature of the Ia bearing cell was not ascertained (Delovitch and McDevitt, 1975).

While it is tempting to suggest that the Ig^{+}, θ^{-}, CR^{-}, $FcR^{\pm}$, $Ia^{\pm}$ cell in foetal liver is equivalent to the accessory cell described in adult spleen the possibility cannot be excluded that its presence is merely coincidental. In foetal tissues Ig^{-} cells may also have accessory cell function as well. The low number of bound SRBC and the instability of the Ig rosettes in foetal liver cell preparations precludes their successful isolation on Isopaque/Ficoll and thus makes their functional characterisation difficult.

The early appearance of accessory cells functional in MLC provides further evidence for the difference between this population and the accessory population that collaborates with T and B cells in the formation of antibody since the latter population does not appear until well after birth (Landahl, 1976).

SUMMARY

In this chapter the stimulator and accessory cell content of foetal liver cells from donors of various ages was studied. From as early as 13 days of gestation allogeneic foetal liver cells were shown to stimulate cytotoxic T cell responses of similar magnitude and specificity to those induced by allogeneic adult spleen cells. Stimulator activity was completely removed following treatment of the foetal liver cells with carbonyl iron. This loss in activity did not appear to be the result of the selective depletion of non-stimulating accessory cells since replacement of these did not restore the response. Stimulator cells, like accessory cells in this system, therefore appeared to belong to a subpopulation of "sticky" cells which represented approximately 30% of the population. One interpretation of the data was that stimulator cells and accessory cells are one and the same in foetal liver. The stimulator population was not further identified but may include mature or immature macrophages or immature B cells all of which are known to be carbonyl iron adherent and present in the populations studied. Foetal liver cells were also noted to become increasingly immunosuppressive after 16 days gestation.

From as early as day 14 of gestation foetal liver cells were also found to be a source of carbonyl iron adherent accessory cells. The possibility of this population being equivalent to the Ig^{+}, θ^{-}, $CR^{\pm}$, $FcR^{\pm}$, Ia^{-} population of accessory cells in adult spleen was discussed. The early appearance of accessory cells during ontogeny provided further evidence for the difference between this population and the one collaborating in antibody formation.

CHAPTER 7

PRODUCTS RELEASED BY CULTURED SPLEEN CELLS CAN SUBSTITUTE FOR ACCESSORY CELLS IN MLC

7.1 Introduction

It has become increasingly evident that direct contact between cells is often not a prerequisite for cellular collaboration. In many instances factors produced by the collaborating cells are the active moieties. For example, T cell derived factors have been described in supernatants from mixed lymphocyte cultures and cultures of cells with T dependent antigens which are capable of replacing helper T cells in both humoral (Dutton *et al.*, 1971; Schimpl and Wecker, 1972; Amerding and Katz, 1974; Taussig *et al.*, 1975), and cell mediated immune responses (Altman and Cohen, 1975; Plate, 1976). Similarly, factors produced by cultured peritoneal cells have been shown to replace the adherent population required for the induction of antibody responses *in vitro* (Möller *et al.*, 1976).

Supernatant factors capable of replacing adherent accessory cells in MLC have also been reported. In an early study Bach and others (1970), described a factor, produced by peritoneal cells in the absence of antigen, which could restore the proliferative response in adherent cell depleted mixed lymphocyte cultures. More recently, Miller and Mishell (1975), observed that the accessory cells required for the induction of cytotoxic T cells *in vitro* could be replaced with a factor produced by spleen cells in the presence or absence of alloantigen.

In this chapter preliminary data is presented which, like that of Miller and Mishell (1975), suggests that cells present in the spleen are capable of producing accessory cell replacing factors when cultured in the absence of alloantigen.

7.2 Methods and materials

7.2.1 Animals

6-8 week old BALB/c and CBA/H mice were used throughout.

7.2.2 Tissue culture techniques

Mixed lymphocyte cultures and cytotoxicity assays were carried out as described in Chapters 2 and 3.

7.2.3 Carbonyl iron treatment

Adherent cells were removed by treatment with carbonyl iron powder as described in Chapter 3.

7.2.4 The separation of cells on the basis of surface Ig.

Spleen cells were separated into Ig^+ and Ig^- subpopulations by the rosetting procedure described in Chapter 3.

7.2.5 Preparation of supernatants

Supernatants were prepared by culturing cells in a final volume of 2 ml in complete medium in 24 well Linbro trays for 18 hours at 37°C in a humidified atmosphere of 7% O_2, 10% CO_2, 83% N_2. After incubation the cultures were harvested and centrifuged at 900 g for 10'. The supernatants were collected and pooled. Supernatants were generally used the day they were harvested although they were found to be stable for at least three weeks if stored at -70°C. The following populations of cells were used to prepare supernatants: 4×10^6 irradiated, untreated or carbonyl iron treated BALB/c or CBA/H spleen cells or 4×10^6 irradiated Ig^+, Ig^- or recombined ($Ig^+ + Ig^-$) BALB/c or CBA/H spleen cells. The same batches of Eagles Minimal Essential Medium (F-15), heat inactivated foetal calf serum and 2-mercaptoethanol were used for all experiments.

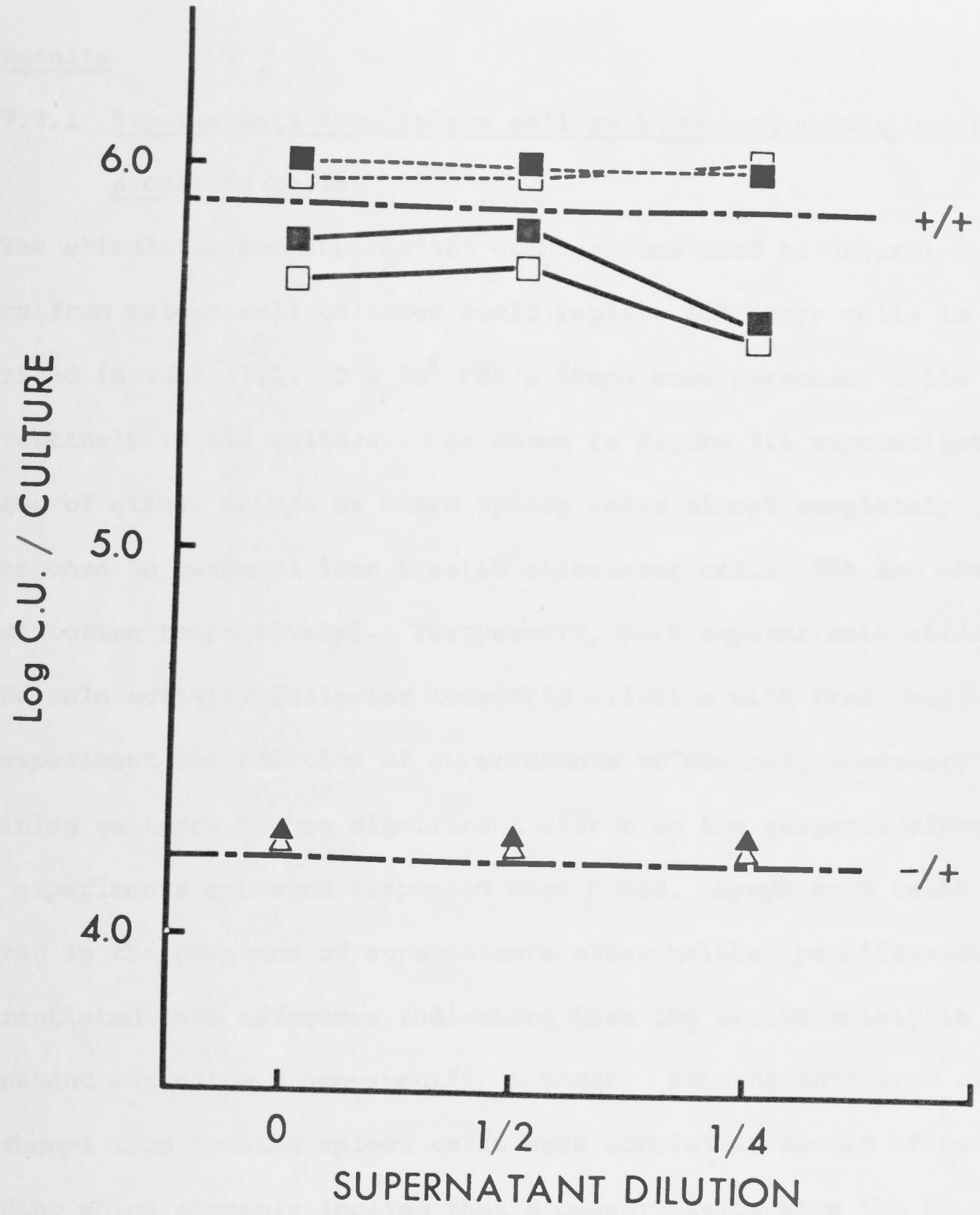

Figure 7.1 - Supernatant factors can replace accessory cells in MLC.

■--■ , BALB/c spleen supernatant + (+/+) culture.

□--□ , CBA/H spleen supernatant + (+/+) culture.

■—■ , BALB/c spleen supernatant + (-/+) culture.

□—□ , CBA/H spleen supernatant + (-/+) culture.

△ , ▲ , supernatants prepared from carbonyl iron treated BALB/c spleens or CBA/H spleens.

+/+, denotes cultures containing 4 x 10^6 irradiated BALB/c spleen stimulator cells + 2 x 10^6 CBA/H lymph node responder cells.

-/+, denotes cultures containing 4 x 10^6 irradiated, carbonyl iron treated BALB/c spleen cells + 2 x 10^6 CBA/H lymph node responder cells.

All supernatants were obtained from overnight cultures of BALB/c or CBA/H irradiated spleen cells.

7.3 Results

7.3.1 Supernatants from spleen cell cultures can substitute for accessory cells

The stimulator and supernatant combinations used to determine whether factors from spleen cell cultures could replace accessory cells in MLC are summarised in Table 7.1. 2×10^6 CBA/H lymph node responder cells were used routinely in all cultures. As shown in Figure 7.1 supernatants from cultures of either BALB/c or CBA/H spleen cells almost completely restored the response to carbonyl iron treated stimulator cells (88% and 69% reconstitution respectively). Furthermore, both supernatants still had considerable activity following four-fold dilution with fresh medium. In this experiment the addition of supernatants to control, accessory cell containing cultures had no significant effect on the response although in other experiments enhanced responses were noted. Lymph node cells cultured in the presence of supernatants alone neither proliferated nor differentiated into effectors indicating that the active moiety in the supernatant was not a a non-specific mitogen. Supernatants from cultures of carbonyl iron treated spleen cells were completely devoid of activity, a finding which strongly implied that accessory cells were the source of the active factor.

It should be stressed, however, that the efficiency with which supernatants reconstituted the response to carbonyl iron treated stimulator cells was extremely variable. Thus as shown in Table 7.2 (line 5) although the addition of supernatants always resulted in a significant increase in the response the degree of reconstitution of the response varied from 6-368%.

7.3.2 The capacity of Ig^+ and Ig^- spleen cells to produce accessory cell replacing factor(s)

In Chapter 5 it was shown that the Ig^+ fraction of spleen cells was a much richer source of accessory cells than the Ig^- fraction. If as

TABLE 7.1

PROTOCOL FOR EXPERIMENTS WITH SUPERNATANTS

Stimulator population[c]		Source of supernatant[b]			
Untreated	Carbonyl iron treated	BALB/c spleen	Carbonyl iron treated BALB/c spleen	CBA/H spleen	Carbonyl iron treated CBA/H spleen
+[a]	-	+	-	-	-
+	-	-	-	+	-
-	+	+	-	-	-
-	+	-	+	-	-
-	+	-	-	+	-
-	+	-	-	-	+

a +, denotes components included in the culture.

b Supernatants were prepared by culturing irradiated cells at a final concentration of 2×10^6/ml, overnight.

c 4×10^6 irradiated BALB/c spleen cells were used as stimulators and 2×10^6 CBA/H lymph node cells as responders throughout.

TABLE 7.2

THE ABILITY OF Ig^+ AND Ig^- SPLEEN CELLS TO PRODUCE ACCESSORY CELL REPLACING FACTOR

Carbonyl iron[a] treatment of stimulator population	Source of[b] supernatant	Experiment No.					
		1	2	3	4	5	6
-[d]	-	5.74	6.17	6.26	5.98	5.98	6.26
+	-	3.88	4.07	5.20	4.00	4.00	5.20
+	Ig^+ spleen cells	N.T.	5.67 (31.6)[c]	4.95 (0)	6.74 (547)	6.63 (452)	5.28 (0)
+	Ig^- spleen cells	N.T.	5.37 (15.8)	4.94 (0)	6.32 (221)	6.29 (200)	5.74 (31.8)
+	Unfractionated spleen cells	5.84 (126)	4.97 (6.3)	6.02 (55)	6.54 (368)	6.51 (337)	5.5 (17.3)
+	$Ig^+ + Ig^-$ spleen cells	N.T.	N.T.	5.50 (17.5)	6.68 (505)	6.54 (368)	5.60 (22.7)

a 4×10^6 irradiated BALB/c stimulator cells and 2×10^6 CBA/H lymph node responder cells were used throughout.

b -, denotes no supernatant. The supernatants used in experiments 1 - 4 were prepared from irradiated BALB/c spleen cells while those used in experiments 5 and 6 were prepared from irradiated CBA/H spleen cells.

c Figures in brackets represent the percentage reconstitution of the response.

d -, no treatment; +, treatment.

N.T., not tested.

suggested in the previous section the accessory cells themselves produce accessory cell replacing factor, supernatants from cultures of Ig^+ cells should be a better source of the factor than supernatants from cultures of Ig^- cells. Supernatants from individual cultures of Ig^+ and Ig^- populations were therefore prepared and compared for their reconstituting activity. As illustrated in Table 7.2 the activity of the supernatants from both populations was very variable. Hence, reconstitution with supernatants from cultured Ig^+ cells ranged from 0-547% while those from cultured Ig^- cells ranged from 0-221%. It was notable, however, that the supernatants from cultured Ig^+ cells in all experiments but one had significantly more activity than those from cultured Ig^- populations. The finding that supernatants from cultured Ig^- cells completely restored the response in two experiments is difficult to explain but possibly reflects either the production of factors by cells other than accessory cells, or a more proportionate distribution of accessory cells between the Ig^+ and Ig^- fractions.

DISCUSSION

In this chapter a factor(s) was described which was capable of replacing accessory cells in MLC. This factor, termed accessory cell replacing factor (ACRF) was produced independently of stimulation by alloantigen by unfractionated spleen cells, Ig^+ and to a lesser extent Ig^- spleen cells of both stimulator and responder origin. A similar accessory cell replacing factor produced by T cell depleted spleen cells cultured in the presence or absence of allogeneic cells has also been described by Miller and Mishell (1975).

Two findings, firstly that carbonyl iron treated cells did not produce ACRF and secondly that Ig^+ cells were generally a better source of the factor than Ig^- cells strongly suggested that accessory cells themselves were responsible for the production of the factor. Furthermore, the fact that ACRF was produced in the absence of stimulation with alloantigen provided further evidence that the fulfilment of accessory cell function requires neither the recognition of nor the processing of antigen.

The complete reconstitution of the response with supernatants from cultured Ig^- cells observed in two experiments deserves further comment. The activity of these supernatants may reflect either the production of T cell or macrophage factors capable of enhancing the response or alternately a more equitable distribution of accessory cells between the Ig^+ and Ig^- fractions. The latter situation could arise if the mice used were overtly stimulated with antigen and as a result had an increased number of immature splenic B cells or macrophages. This is not a remote possibility since the animals used in these studies are bred under specific pathogen free conditions but just prior to use are maintained under conventional conditions and are therefore more susceptible to mild infections.

The extreme variability in the activity of the various supernatants tested may also be explained if only spleens from animals recently stimulated with antigen contained sufficient numbers of accessory cells to produce appreciable amounts of ACRF *in vitro*. The use of spleen cells from recently immunised mice for the preparation of supernatants may help to resolve this point. When accessory cells themselves rather than ACRF are used to restore the response in adherent cell depleted cultures the immune status of the animals is probably less critical since fewer accessory cells would be required to reach the required local concentration of ACRF than would be required to produce a similar concentration in a supernatant.

The preliminary nature of the data described in this chapter precludes any positive statements on the target for ACRF or its mode of action. Kasakura and Lowenstein (1965) and Kasakura (1970, 1976) have described blastogenic factors produced by lymphocytes cultured in the absence of antigen which induced the proliferation of both syngeneic and allogeneic lymphocytes. ACRF does not seem to fit into this category, however, since lymph node responder cells cultured in the presence of supernatants for 5 days neither proliferated nor differentiated into effector cells. Alternative possibilities are that ACRF only induces proliferation in responder cells after they have bound antigen or that it acts at stimulator cell rather than responder cell level. These alternatives will be elaborated on in the following chapter.

Clearly, there are many experiments yet to be done before the identity of ACRF and its mode of action can be elucidated. Some of these include: the physical and biochemical characterisation of the factor(s); pre-incubation of stimulator or responder populations with ACRF to determine the target cell for ACRF activity; and determination of the conditions required for maximal production of ACRF *in vitro*. It will also be interesting to see whether L cells which can function as both stimulator and accessory cells produce factors resembling ACRF.

S U M M A R Y

In this chapter very preliminary data was presented which suggested that accessory cells could be replaced by a factor(s) termed accessory cell replacing factor (ACRF) which was produced by spleen cells of either responder or stimulator cell genotype cultured in the absence of antigen. Two further observations, firstly that supernatants from cultures of Ig^+ spleen cells were generally a better source of ACRF than cultures of unfractionated spleen cells or Ig^- spleen cells and, secondly that carbonyl iron treated cells did not produce ACRF, suggested that accessory cells were responsible for the production of ACRF. The fact that ACRF did not induce the proliferation of responder lymph node cells implied that it did not function simply as a T cell mitogen. Whether ACRF acts at the level of the stimulator or the responder population was not resolved.

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

The aim of this work was to identify and characterise the cells which interact in the stimulation of cytotoxic T cell responses to alloantigens *in vitro*. Interpretations of the individual findings have been given at the end of each chapter. In this concluding section further implications of the findings are discussed and a model is proposed which attempts to explain how the components of the system, viz., stimulator cells, accessory cells, and precursors of cytotoxic T cells interact.

8.1.1 Synopsis of observations

Although few comparative studies of the stimulatory capacity of different cell populations exist, it can be concluded from the data in Tables 1.2, 3.1 and 3.2 that in mice and humans lymphocytes and some neoplastic cells generally stimulate stronger cytotoxic T cell responses than mature macrophages or non-lymphoid cells.

Ontogenic studies and studies with adult lymphoid populations provided evidence that stimulator activity was a function of both mature and immature lymphoid cells and was not dependent on the presence of surface Ig, Ia antigens or Fc or complement receptors. Furthermore, significant cytotoxic T cell responses were obtained in the absence of I region differences between stimulator and responder populations, providing further evidence that helper T cells which recognise Ia antigens, are not an absolute requirement for the induction of cytotoxic T cell responses *in vitro*.

When mature non-adherent resting lymphocytes were used as a source of stimulator cells in primary MLC there was an additional and absolute requirement for an adherent accessory cell population. This cell population was present predominantly in spleen, bone marrow and foetal liver, and to a lesser extent in lymph nodes and the peritoneal cavity, but was absent from

the thymus. The accessory cells were required for the induction rather than the maintenance of the response, functioned whether syngeneic or allogeneic with the responder population and were completely removed by treatment with carbonyl iron. Moreover, the observation that accessory cells could be replaced with L cells and factors produced by lymphoid cells cultured in the absence of antigen, suggested that this population featured in neither the presentation nor the processing of antigen and thus differed from the accessory cell population required for the induction of antibody responses *in vitro*.

The splenic accessory cell was further characterised as an $Ig^{+}\theta^{-}$ and tentatively $FcR^{\pm}$, $CR^{\pm}$, Ia^{-} cell which was not a mature macrophage. This data together with indirect evidence from ontogenic studies implied that the functional population was comprised predominantly of immature B cells, although the possibility that some accessory cells were immature FcR^{+} macrophages was not completely eliminated.

Although accessory cells were required for the induction of primary responses when resting lymphocytes were the source of stimulators this requirement was less critical when neoplastic stimulator cells or memory responder populations were used.

The induction of a primary cytotoxic T cell response *in vitro* therefore appeared to require a minimum of three cell types: lymphoid stimulator cells, $Ig^{+}\theta^{-}FcR^{\pm}CR^{\pm}Ia^{-}$ accessory cells and precursor cytotoxic T cells. How these individual populations interact with each other is unknown at present and can only be speculated upon.

8.1.2 A model for T cell induction

A. Considerations - An adequate model must account for at least some of the following facts:

(1) Serologically defined MHC antigens alone, either in purified form, on subcellular membrane fragments or presented on the surface of non-lymphoid cells are insufficient stimuli for

the induction of primary cytotoxic T cell responses (Lafferty *et al.*, 1969, 1972, 1974a, 1975, 1976; Rode and Gordon, 1974; Engers *et al.*, 1975b and Wagner *et al.*, 1976a).

(2) Membrane fragments and UV irradiated, glutaraldehyde fixed and heat (45°C) treated cells all induce strong secondary cytotoxic T cell responses but not primary responses (Röllinghoff and Wagner, 1975; Engers *et al.*, 1975b; Wagner *et al.*, 1976a; Häyry and Andersson, 1976).

(3) The primary response to non-stimulating, UV irradiated or heat treated spleen cells can be restored by the addition of third party stimulator cells (Lafferty *et al.*, 1974a; Schendel and Bach, 1974, 1975).

(4) Glutaraldehyde fixed lymphoid cells are able to stimulate primary responses if they are activated with mitogens prior to fixation (Lightbody and Kong, 1976).

(5) Stimulator cells must be metabolically active and possibly capable of synthesising proteins (Wagner, 1973).

(6) Helper T cells activated by H-2I or Mls loci coded antigens are not an absolute requirement for cytotoxic T cell induction (Nabholz *et al.*, 1974; Klein *et al.*, 1975b; Forman and Klein, 1975 ; Melief *et al.*, 1975; Hodes *et al.*, 1976).

(7) When resting non-adherent lymphocytes are used as a source of stimulators there is an absolute requirement for an adherent accessory population (Wagner *et al.*, 1972; MacDonald *et al.*, 1973b, Chapters 4 and 5).

Taken together these observations suggest that critical inductive signals not supplied by antigen alone are required for the induction of primary cytotoxic T cell responses. Similar conclusions have also been

reached by Lafferty and Cunningham (1975) who proposed a model for cytotoxic T cell induction, based on the original Bretscher/Cohn 2 signal model for B cell induction (Bretscher and Cohn, 1970), where the stimulator cell population provided both the antigenic stimulus (signal 1) and the additional inductive stimulus (signal 2). Bach *et al.* (1976) have also proposed a 2 signal model which differs from that of Lafferty and Cunningham (1975), in that the second signal is provided by a helper T cell rather than the stimulator cells.

The data obtained with LPS activated and therefore possibly Ia^+ fixed stimulator cells (Lightbody and Kong, 1976) and more particularly UV treated stimulator cells (Lafferty *et al.* 1974a; Schendel and Bach, 1975) and heat treated cells (Schendel and Bach, 1974) indicate that in some instances the second inductive signal may come from sources other than stimulator cells, probably helper T cells. Recent studies have indeed suggested that factors produced by T cells can stimulate cytotoxic T cell precursors (Altman and Cohen, 1975; Plate, 1976). However since helper T cells, especially those recognising Ia antigens and Mls antigens, are not normally an absolute requirement for cytotoxic T cell induction they are probably not the prime source of signal 2 in mixed lymphocyte cultures.

Whether two signals - one resulting from antigen recognition and the other deriving from stimulator cells (or some other source) - are, in fact, required for T cell induction, or alternatively a single inductive signal suffices is as contentious an issue for T cells as it is for B cells (Bretscher and Cohn, 1970; Coutinho and Möller, 1974; Möller *et al.*, 1976). For the sake of simplicity the inductive signal will also be referred to as signal 2 in the model to be described here.

If stimulator cells, as proposed by Lafferty and Cunningham (1975), provide both signals for T cell induction the presence of an accessory cell population would appear to be superfluous. However, the abrogation

of the response following the removal of accessory cells and their apparent active participation in induction suggests a vital role for this population. Accessory cell activity may be manifested in two possible ways:

(1) Accessory cells - a unique subpopulation of lymphocytes - supply inductive signals directly to responder cells.

(2) Accessory cells activate lymphocytes in such a way that the lymphocytes are then able to deliver signal 2. Thus, accessory cells may convert resting lymphocytes to stimulator cells, implying that only activated lymphocytes are capable of delivering signal 2.

The inherent capacity to supply inductive signals is by no means unique to cells resembling accessory cells. Apart from helper T cells, there is also evidence that blast cells and neoplastic cells can directly stimulate cytotoxic T cell responses. Thus, when the stimulator activity and accessory cell requirements of T lymphoblasts obtained from mixed lymphocyte cultures and unstimulated T lymphocytes were compared, the former population was observed to induce stronger cytotoxic responses and to be less accessory cell dependent that the latter (Lafferty and Woolnough, 1977). Similarly P815, L-929 and EL-4 cells, all of which are rapidly dividing neoplastic cell lines and probably perpetually in a "blast-like" state, are capable of inducing significant cytotoxic T cell responses in the absence of accessory cells (Chapter 5 and Lafferty and Woolnough, 1977). Blast-like cells, unlike resting lymphocytes, therefore appear to have the ability to independently transmit inductive stimuli to precursor cytotoxic T cells.

The fact that accessory cells exhibit few of the surface markers normally associated with mature B cells or macrophages and are present in appreciable quantities in haemopoietic organs, such as bone marrow and foetal liver, suggests that they too may be "activated" or "blast-like" in nature and, as a consequence, may be able to interact directly with responder

T cells. Thus, when syngeneic to the responder population accessory cells may function as a source of inductive stimuli for cytotoxic T cell precursors while cells in the accessory cell depleted stimulator population provide a source of antigen. In such a system lymphocytes would merely function as inert vehicles of transplantation antigen and it could be predicted that any source of MHC antigen would suffice for induction provided adequate numbers of accessory cells were available. However, antigen presented in soluble or subcellular form fails to stimulate a response and several lines of evidence suggest that lymphocytes in the stimulator population play an active role in the inductive process. Thus, lymphocytes treated with metabolic inhibitors, or inactivated by fixation, UV irradiation or heat treatment all fail to stimulate primary responses although they still exhibit antigens in a form recognisable by memory cells (Röllinghoff and Wagner, 1975, Häyry and Andersson, 1976). The inability of the UV treated cells to stimulate is not the result of the simultaneous inactivation of accessory cells since the addition of irradiated responder spleen cells to cultures of UV treated stimulator cells and untreated responder cells (equivalent to the three cell system described in Chapter 5) did not restore the response Lafferty *et al.* 1974a). A further interpretation of the latter observations is that accessory cells do not provide substantial direct stimuli to responder cells. The possibility cannot be excluded, however, that for some obscure reason only viable, intact cells can present antigen in an immunogenic way to unprimed T cells and that inductive stimuli from accessory cells are directed to responder cells.

A more attractive system is one where accessory cells activate resting lymphocytes in the stimulator population and convert them to "stimulator cells". Such a system would explain both the apparent active involvement of stimulator cells in the inductive process and the requirement for accessory cells.

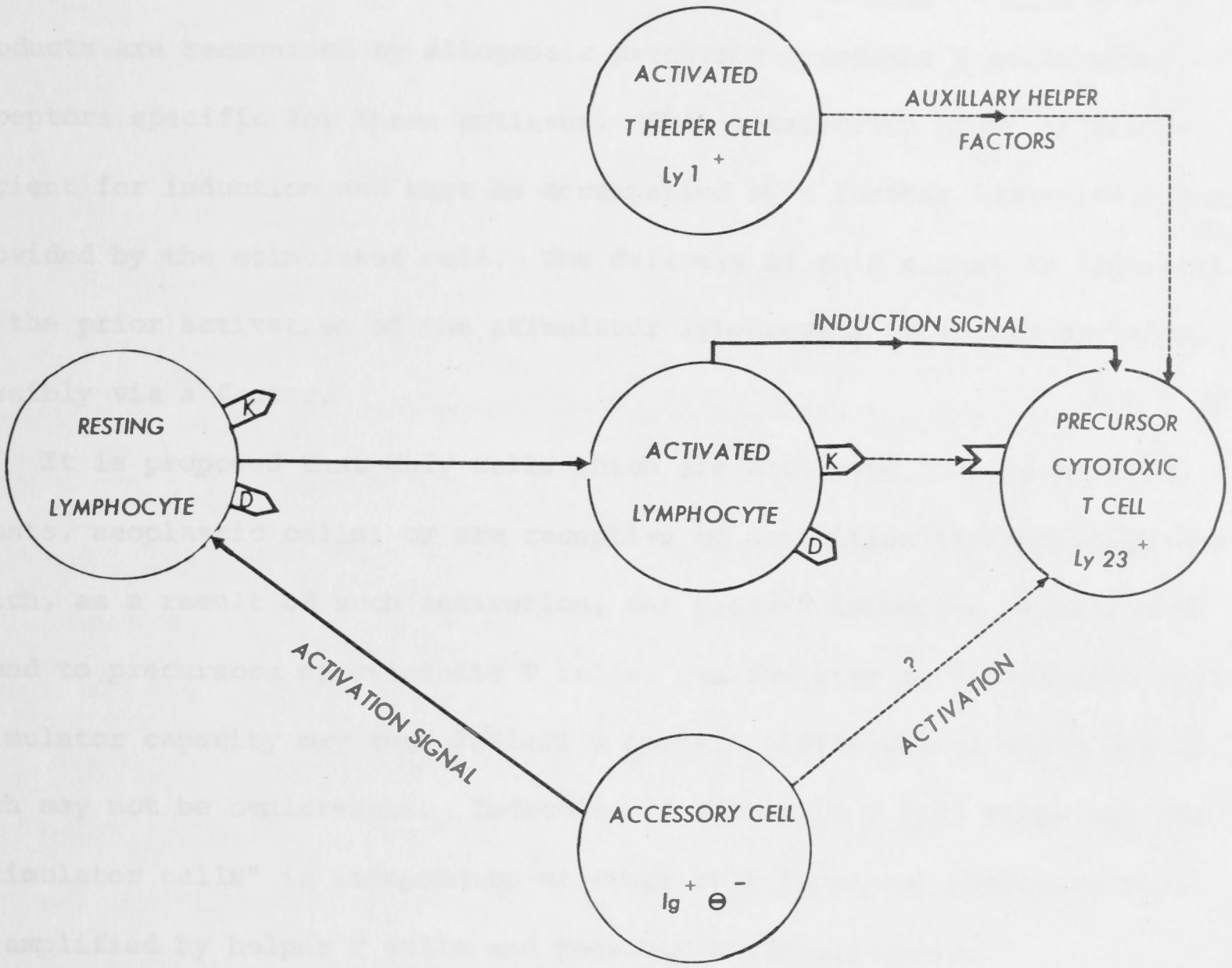

Figure 8.1 - Diagrammatic representation of the cellular interactions proposed to occur during the induction of cytotoxic T cell response *in vitro*. Induction requires both the recognition of K or D antigens by Ly 2-3$^+$ precursor cytotoxic T cells and the receipt of an inductive signal provided by activated stimulator cells or possibly accessory cells. Responses may be further amplified by factors from Ly-1$^+$ helper T cells stimulated by Ia or Mls antigens.

B. The model - Figure 8.1 summarises the cellular interactions thought to be required for the generation of cytotoxic T cell responses in mixed lymphocyte cultures. Stimulator cells bearing H-2K or H-2D gene products are recognised by allogeneic precursor cytotoxic T cells with receptors specific for these antigens. This interaction alone is insufficient for induction and must be accompanied by a further inductive signal provided by the stimulator cell. The delivery of this signal is dependent on the prior activation of the stimulator lymphocytes by accessory cells, possibly via a factor.

It is proposed that only cells which are activated (immature cells, blasts, neoplastic cells) or are receptive to activation (lymphocytes) and which, as a result of such activation, can produce inductive stimuli when bound to precursors of cytotoxic T cells, can function as "stimulator cells". Stimulator capacity may thus reflect a certain physiological state and as such may not be omnipresent. Induction of cytotoxic T cell responses via "stimulator cells" is independent of other cell types but responses may be amplified by helper T cells and possibly accessory cells.

Non-lymphoid cells bearing Ia antigens such as epithelial cells may circumvent the necessity of supplying inductive stimuli themselves by activating helper T cells which then provide an alternative source of inductive signals. Thus, following recognition of antigen, cytotoxic T cell precursors may be induced to proliferate by stimuli from activated stimulator cells or helper T cells.

It should be emphasised that while the model illustrated in Figure 8.1 may explain the responses observed in the three-cell system described in Chapter 5, such a mechanism may in fact only represent an amplification process in a conventional MLC. If accessory cells are in fact "stimulator cells" they may provide the primary source of stimulation in untreated spleen cell stimulator populations. Accessory cells of either stimulator or responder origin would have the additional capacity to recruit further

"stimulator cells" from the resting lymphocyte pool by producing "activation factors". Accordingly accessory cells may be both "stimulators" and recruitors of "stimulators". Characterisation of the supernatants described in Chapter 7 and the isolation of accessory cells will help to resolve these issues.

8.1.3 The nature of signal-2

The nature and the mode of action of the inductive stimulus or "signal-2" is unknown. One possibility is that it takes the form of a signal to proliferate. Evidence in support of this suggestion comes from studies with memory populations where it has been shown that the differentiation of memory cells into secondary effectors is relatively independent of both accessory cells (Chapter 6) and proliferation (MacDonald *et al.*, 1975; Wagner and Röllinghoff, 1976).

Alternatively, inductive signals may ensue when antigens on the surface of "stimulator cells" align with their respective receptors on the surface of responder T cells. The recent report that fixed cells could stimulate provided they were activated prior to fixation (Lightbody and Kong, 1976) suggests that the recognition of antigens present on the surface of blast cells may indeed be sufficient stimulus for induction.

8.1.4 Postulates on stimulator antigens and T-cell receptors

It is worth noting that the recognition of a single antigen on a stimulator cell by a single receptor on the responding T cell as illustrated in Figure 8.1 may prove to be an oversimplification. Simultaneous recognition of membrane antigens in association with or in addition to K or D antigens by one or more receptors may in fact be a prerequisite for the induction of cytotoxic T cell responses to alloantigens. As mentioned in Chapter 1 a variety of systems along these lines have already been suggested for the induction of cytotoxic T cells with specificity for virus-infected or chemically modified syngeneic or allogeneic cells (Zinkernagel and

Doherty, 1974; Shearer, 1974; Blanden *et al.*, 1975; Zinkernagel, 1976; von Boehmer and Haas, 1976).

Recently Janeway *et al.*(1976) proposed a model for a T cell receptor system which involved two intimately associated receptors one with specificity for non-MHC antigens and the other with specificity for alloantigens and low affinity for self MHC antigens. It was suggested that the induction of responses by T cells with specificity for antigens expressed on the surface of self cells required the occupation of both receptors whereas the induction of responses by T cells recognising alloantigens required only the binding of the receptor specific for alloantigen.

It is possible, however, that a uniform T cell receptor system operates and that the stimulation of allo-reactive T cells also requires the recognition of more than one antigen by two independent receptors. The second antigen could take the form of minor histocompatibility antigens or may be represented by part of the K or D antigens. In the latter instance a "hapten-carrier" type of arrangement could be envisaged where each receptor recognises a different determinant on the same molecule. Since the minor H antigens are highly polymorphic and are known to be recognised in association with K or D antigens (Bevan, 1975) they may form the bulk of the non-MHC antigens recognised with K or D antigens. Thus a subset of T cells which all have "MHC" receptors specific for one particular alloantigen but "non-MHC" receptors specific for any number of minor H antigens would greatly expand the "apparent" response to any one alloantigen. Such a system would explain the apparently high proportion of T cells responding to alloantigens equally as well as that proposed by Janeway *et al.* and has the additional advantage that it makes no awkward demands on the specificity or affinity of the receptors for MHC antigens.

The reason that activated lymphocytes and neoplastic cells stimulate stronger cytotoxic T cell responses than other cells may be a reflection of the type and the concentration of antigen expressed on their surfaces.

Accordingly, only intact and activated cells may express stable complexes of MHC and non-MHC antigens in sufficient concentration to induce an appreciable primary response. In contrast, the concentration of antigenic complexes may be less critical for the restimulation of memory effector T cells and would explain why these cells are stimulated by subcellular antigens, fixed and UV treated resting lymphocytes and non-lymphoid cells.

8.1.5 *In vivo* correlates

The foregoing highly speculative discussion was based almost entirely on data obtained from *in vitro* experiments. It may well be asked what relationship the cells required for the induction of immune responses, *in vivo*, to allografts and tumours bear to those described *in vitro*.

As already discussed at some length in Chapter 1 it is generally agreed that cytotoxic T cells play a prominent role in the rejection of allografts and tumours. Although some exceptions do exist there is considerable evidence that the removal of passenger leukocytes from donor skin (Summerlin *et al.*, 197), kidney (Stuart *et al.*, 1971) and thyroid (Lafferty *et al.*, 1975, 1976) allografts results in a severe depression of their immunogenicity and the consequent prolongation of their survival in the recipient. These observations imply two things: first, that lymphoid cells are superior stimulators *in vivo* as well as *in vitro* and second, that transplantation antigens carried on tissue cells need not present the major barrier to allo-transplantation.

There is also evidence that helper T cells activated by Ia antigens are not an absolute requirement for the rejection of allografts although an I region difference between the donor and the recipient certainly accelerated the rejection process (Sondel and Bach, 1976; Wagner *et al.*, 1976b). Helper T cells therefore appear to perform as an amplifier population both *in vitro* and *in vivo*. The role of accessory cells *in vivo* is unknown and may be extremely difficult to establish since they can be of either donor or recipient origin.

To conclude, there is clearly still much to be done in elucidating the mechanisms by which cytotoxic T cells are generated both *in vitro* and *in vivo*. Verification or amendment of the model proposed here awaits further studies on stimulator, accessory and helper T cell populations and ultimately rests on the characterisation of both the antigens recognised by T cells and the receptors used to recognise them.

APPENDIX I

A PROCEDURE FOR REMOVING RED CELLS AND DEAD CELLS FROM LYMPHOID CELL SUSPENSIONS

Introduction

It is important that efficient methods are available for removing dead cells and red cells from lymphoid cell suspensions as such cells frequently interfere with immunological assays. For example, red cells can inhibit mixed lymphocyte cultures (MLC) and dead cells can non-specifically bind proteins and thereby give high backgrounds in antigen-binding studies. In accurately assessing cytotoxic antisera it is also important that there is initially a low percentage of dead cells in the cell suspensions being examined. Furthermore, if dead cells can be easily eliminated, sequential cytotoxicity assays with different antisera become possible.

Two methods have already been published which can be used to eliminate dead cells and red cells from lymphoid cell suspensions. One procedure separates viable cells from red cells and damaged cells by centrifuging the mixture on albumin density gradients (Shortman *et al.*, 1972c). By varying the pH of the albumin gradient different separations were achieved, i.e. at pH 5.1 cells killed by antibody and complement were not removed whereas at pH 7.2 such cells were depleted. The second method is based on the observation that dead cells aggregate and adhere to surfaces in media of low ionic strength (von Boehmer and Shortman, 1973). This procedure is technically simpler than the albumin gradient method, but suffers from the disadvantage that dead cells cannot be eliminated from all cell suspensions and there is no depletion of red cells. This paper describes an additional red cell and dead cell removal procedure which is generally more versatile than the previously reported methods.

Methods and materials

Animals

CBA/H and BALB/c mice of either sex and from 6-10 weeks of age were used.

Immunization and irradiation of mice

CBA/H mice were primed intraperitoneally 1-3 months before use with 500 µg of alum-precipitated dinitrophenol-bovine serum albumin (DNP-BSA), mixed with 10^9 *Bacillus pertussis* organisms (Commonwealth Serum Laboratories, Melbourne) in 0.2 ml saline. Recipient CBA/H mice were given 850 rads whole body γ-irradiation from a ^{60}Co source at 40-50 rads/min, 24 hr before cell transfer and challenge with antigen. In transfer experiments, irradiated recipients received intravenously 5 µg of DNP-MON and 10^7 viable lymphocytes in 0.5 ml of phosphate buffered saline (PBS)/ 10% foetal calf serum (FCS).

Antigens and antisera

Monomeric flagellin (MON), mol. wt 40,000, was prepared from the flagella of *Salmonella typhimurium* SL 870 (Ada *et al.*, 1964), and oxidized to prevent repolymerization (Parish and Stanley, 1972). The MON was dinitrophenylated according to the method of Eisen (1964), and a preparation of MON conjugated with approximately 1.5 DNP groups/mole used.

A cytotoxic anti-θ ascitic fluid was prepared in AKR/J mice as reported elsewhere (Kirov, 1974).

Preparation of lymphoid cells

Two procedures were used to obtain cell suspensions from spleen, thymus and lymph node. The first procedure was carried out at 0-4°C and entailed cutting the lymphoid organs into small segments, extruding the segments through a stainless steel sieve into PBS/10% FCS and then washing the extruded cells twice in PBS/10% FCS by centrifugation at 4°C. This method yielded cell suspensions with viabilities ranging from 55-70%. The second procedure resulted in cell suspensions with much

higher viabilities (85-95%) and has been described in detail previously (Parish *et al.*, 1974). Briefly, the procedure consisted of preparing the cell suspensions at room temperature (15-20°C) and removing large aggregates and fine debris by multiple centrifugations of the cells at low g-forces.

Cortisone-resistant thymus cells were obtained from CBA/H mice which had received 3 mg of cortisol intraperitoneally 24 hr before sacrifice. These thymus cells were prepared at 0-4°C as described above.

Total viable and dead cells in each lymphoid cell suspension were counted in a haemocytometer using the Trypan blue exclusion method. In this procedure 0.1 ml of the cell suspension in PBS/10% FCS was mixed with 0.1 ml of 0.1% (w/v) Trypan blue in PBS, and the proportion of unstained cells counted 1-2 min later.

When cells were prepared for tissue culture, they were collected and washed in Eagle's minimal essential medium (F-15) (Grand Island Biological Co., Grand Island, New York) buffered with sodium bicarbonate and supplemented with 10% FCS, 100 µg/ml streptomycin, 100 units/ml penicillin G and 10^{-4} M mercaptoethanol.

Enumeration of anti-DNP plaque-forming cells (PFC)

PFC were enumerated using the technique of Cunningham and Szenberg (1968). To detect anti-DNP PFC, sheep erythrocytes were coated with dinitrophenylated rabbit anti-sheep erythrocyte Fab (Strausbauch *et al.*, 1970). The tissue culture response was largely IgM in nature so only direct PFC were assayed. In the *in vivo* experiments both direct and indirect PFC responses were estimated, indirect PFC being developed by a rabbit antimouse Ig antiserum.

In vitro culture of lymphoid cells

The mixed lymphocyte cultures (MLC) consisted of incubating γ-irradiated BALB/c spleen cells (4×10^6/ml) with CBA/H lymph node cells (2×10^6/ml) and measuring cytotoxic activity after 4 days of culture (Lafferty *et al.*, 1974). Cytotoxic cells specific for the H-2^d antigen (i.e. BALB/c) were assayed against ^{51}Cr-labelled P815 mastocytoma (H-2^d) target cells (Brunner *et al.*, 1968).

The *in vitro* anti-DNP response to DNP-MON was produced by culturing CBA/H spleen cells (5×10^6 cells in 2.5 ml) in 16 mm Linbro trays (Linbro Chemical Co., New Haven, Connecticut, USA) as described previously (Kirov, 1974).

Procedure for removal of red cells and dead cells

To 5.0 ml of PBS/10% FCS or tissue culture medium containing from 10^7 to 5×10^8 lymphocytes was added 20 µl of 25% (w/v) sodium azide (final concentration 0.1%) and the mixture was then brought to 20°C by incubation for 3 min in a 37°C water bath. This preparation was layered gently onto 4.0 ml of separating medium which had also been prewarmed to 20°C and which consisted of 12 parts of 14% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) dissolved in distilled water and 5 parts of 32.8% (w/v) sodium metrizoate (Isopaque; Nyegaard and Co., Oslo, Norway), the complete mixture containing 0.1% (w/v) sodium azide. This mixture had a density of 1.09, was stored at 4°C, protected from light and will be referred to as Isopaque/Ficoll. It was easily sterilized by Millipore filtration.

The separations were carried out either in 12 ml, U-bottomed, polycarbonate centrifuge tubes (16 x 100 mm; cat. No. 272; Ivan Sorvall Inc., Norwalk, Connecticut, USA) or in freshly siliconized glass tubes of similar dimensions. After the layering of the lymphoid cell suspension on the Isopaque/Ficoll, the tubes were placed in a centrifuge prewarmed to 20°C and spun at 2000 g (at the Isopaque/Ficoll interface) for 15 min.

The best depletions of red cells and dead cells were obtained if the centrifuge accelerated rapidly and attained 2000 g within 20 sec. After centrifugation the supernatant above the Isopaque/Ficoll interface was discarded and the white cell layer at the interface, together with all the separating medium above the red cell-dead cell pellet, collected. The white cell preparation was then diluted with 10 ml of cold medium (either PBS/10% FCS or tissue culture medium), mixed and the white cells pelleted by centrifugation at 300 g for 10 min at 4°C. The pelleted cells were then washed once more with medium and counted.

Results and discussion

Effectiveness of procedure

Table 1 summarises the efficiency of the Isopaque/Ficoll procedure in removing red cells and dead cells from different populations of mouse lymphoid cells. It can be seen that the procedure very effectively removes both red cells and dead cells from spleen, lymph node and thymus cell suspensions. Furthermore, high recoveries of viable cells were usually obtained. In subsequent experiments it was found that red and dead cells were also eliminated from bone marrow and foetal liver cell suspensions by this technique.

The method was equally effective at depleting red cells and dead cells from lymphoid cell suspensions of either high or low viability. However, when $>10^8$ nucleated cells were applied to the Isopaque/Ficoll, the recovery of viable cells declined. In subsequent experiments no more than 10^8 nucleated cells were applied to each separation tube.

Some technical aspects of the separation procedure should be noted at this point:

(a) First, the nature of the medium in which the cells were suspended did not significantly influence the separation efficiency. Similar results were obtained whether the cells were applied in PBS, PBS/10% FCS or F-15.

TABLE 1

REMOVAL OF RED CELLS AND DEAD CELLS FROM DIFFERENT POPULATIONS OF MOUSE LYMPHOID CELLS

Cells fractionated	Initial viability (%)	Viability after procedure (%)	Recovery of viable cells (%)	Depletion of red cells (%)
10^7 spleen cells	66.2	95.1	100	>99.8
3×10^7 spleen cells	66.2	97.4	100	>99.8
10^8 spleen cells	66.2	99.7	100	98.2
10^8 spleen cells	89.0[a]	99.0	99.0	99.8
2×10^8 spleen cells	66.2	92.8	85.3	99.0
5×10^8 spleen cells	66.2	96.8	72.2	99.3
10^8 lymph node cells	62.0	>99.8	95.7	>99.8
10^8 cortisone-resistant thymus cells	58.0	99.5	82.0	99.0

a Spleen cell suspension prepared at 20°C. All other cell suspensions prepared on ice (see Methods and materials). Cell numbers refer to total number of nucleated cells applied.

TABLE 2

REMOVAL OF SPLEEN CELLS KILLED BY CYTOTOXIC ANTISERA

Spleen cell treatment	Initial viability (%)	Viability after procedure (%)	Recovery of viable cells (%)
10^8 cells treated with normal ascitic fluid and complement			
Expt. 1	98.8	99.8	92.0
Expt. 2	95.7	98.9	98.8
10^8 cells treated with anti-θ ascitic fluid and complement			
Expt. 1	66.6	98.7	83.2
Expt. 2	69.5	97.7	98.5

Spleen cell suspensions prepared at 20°C prior to treatment with ascitic fluids.

(b) In contrast, the presence of sodium azide was essential if good separations were to be achieved. It was noted that sodium azide was somewhat labile and so a fresh stock solution was prepared every 4-6 weeks. It is not known why sodium azide has such a marked influence on the efficiency of separation.

(c) The separations were performed either in poly-carbonate centrifuge tubes or freshly siliconized glass centrifuge tubes as white cells did not adhere to the walls of these tubes. As much as 30-40% of the applied cells were lost during the procedure if untreated glass tubes were used.

The results presented so far demonstrate that the Isopaque/Ficoll procedure is very effective at removing dead cells from cell suspensions teased out from lymphoid organs. Cell death in these cell suspensions is probably due to mechanical stress. Several other experiments were carried out to determine whether cells killed by other methods, such as by the action of cytotoxic antisera and complement, or by several days in tissue culture, could also be removed by the Isopaque/Ficoll procedure.

Table 2 summarizes the ability of the procedure to remove cells killed by anti-θ antiserum and complement. In two separate experiments it was found that antiserum-treated cell suspensions could be completely cleared of dead cells by the Isopaque/Ficoll procedure, with good recovery of viable cells. After dead cell removal >1% of the anti-θ-treated cells were killed when they were re-exposed to anti-θ antiserum. In contrast, anti-θ killed the expected percentage of cells in the population treated with normal serum. Furthermore, there was a substantial enrichment of Ig-bearing cells in the anti-θ-treated population following dead cell removal, i.e. from 45-50% Ig^+ cells in untreated spleen to 65-80% Ig^+ cells in anti-θ-treated spleen.

TABLE 3

REMOVAL OF DEAD CELLS FROM MIXED LYMPHOCYTE CULTURES (MLC)

Cells fractionated	Initial viability (%)	Viability after procedure (%)	Recovery of viable cells (%)
2×10^7 cells from MLC	41.8	96.4	96.3
3.8×10^7 cells from MLC	43.3	95.9	89.7

MLC consisted of incubating γ-irradiated BALB/c spleen cells with CBA/H lymph node cells. The cells were harvested and fractionated after 4 days of culture.

TABLE 4

RECOVERY OF CYTOTOXIC CELLS AFTER DEAD CELL REMOVAL PROCEDURE

Cells fractionated	Cytotoxic activity[a] log C.U./culture		Recovery of cytotoxic activity (%)
	Before fractionation (%)	After fractionation (%)	
2×10^7 cells from MLC	6.73	6.68	89.2
3.8×10^7 cells from MLC	6.73	6.60	74.1

Cytotoxic cells were generated by incubating γ-irradiated BALB/c spleen cells with CBA/H lymph node cells. The cells were harvested and depleted of dead cells after 4 days of culture. The cell preparations tested for cytotoxic activity were the same as those used in the tests shown in Table 3.

a Cytotoxic activity assayed on ^{51}Cr labelled P815 cells. Background = 4.40 log C.U.

It was also found that the Isopaque/Ficoll procedure very efficiently depleted dead cells from cell suspensions which had been subjected to several days of tissue culture (Table 3). The viable cells were also recovered in high yield.

Effect of procedure on immunological activity of cells

The immunological activity of cell suspensions which had experienced the Isopaque/Ficoll procedure was tested in several ways. Initially it was shown that there was no selective loss of either θ^+(T) or Ig^+(B) cells during the separation procedure (Parish *et al.*, 1974). Applying a stringent test of the immunological function of T cells, the ability of cytotoxic T cells to survive the procedure was determined (Table 4). It was found that the cytotoxic cells were recovered in good yield (>80%) although there were indications that as more nucleated cells were applied to the Isopaque/Ficoll, there was a steady decline in the recovery of cytotoxic activity. In additional experiments it was found that the precursors of the cytotoxic T cells were also recovered in high yield (Parish *et al.*, 1974).

The immunological function of helper T cells and antibody-forming cell precursors (B cells) following the separation procedure was assessed both *in vivo* and *in vitro* by measuring the antibody response to the thymus-dependent antigen DNP-MON. In three separate experiments removal of red cells and dead cells had little or no effect on the *in vitro* antibody response to DNP-MON (Table 5). A similar result was obtained with the *in vivo* antibody response (Table 6), in this case both direct and indirect PFC responses being not significantly affected by the separation procedure.

TABLE 5

EFFECT OF DEAD CELL REMOVAL PROCEDURE ON ANTIBODY RESPONSE OF MOUSE SPLEEN CELLS *IN VITRO*

Cell preparation	Direct anti-DNP PFC/culture[a]		
	Expt. 1	Expt. 2	Expt. 33
Unfractionated spleen cells	565 ± 59[b]	1193 ± 117	1303 ± 87
Spleen cells after red and dead cell removal	677 ± 41	1048 ± 38	1120 ± 68

a Spleen cells (5×10^6 viable cells in 2.5 ml) cultured with 100 ng/ml of DNP-MON. PFC assays made after 3 days of culture.

b Standard error of mean of four cultures.

TABLE 6

EFFECT OF DEAD CELL REMOVAL PROCEDURE ON ANTIBODY RESPONSE OF MOUSE SPLEEN CELLS *IN VIVO*

Cell preparation	Anti-DNP PFC/spleen[a]	
	Direct	Indirect
Unfractionated spleen cells	2308 ± 216[b]	3648 ± 640
Spleen cells after red and dead cell removal	2408 ± 384	4332 ± 1330

a 10^7 viable DNP-BSA-primed spleen cells were injected into each mouse with 5 μg of DNP-MON. The mice received 850 rads of γ-irradiation 24 hr prior to cell transfer. PFC assays made 6 days after cell transfer.

b Standard error of mean of five recipients.

Comparison with other procedures

The Isopaque/Ficoll procedure for removing red cells and dead cells from lymphoid cell suspensions has several distinct advantages over previously described techniques. A major advantage is that the procedure is much more versatile than any previously reported method, being able to remove red cells and dead cells from a wide range of lymphoid cell preparations and simultaneously achieve high recoveries of viable cells. In contrast, when albumin gradients are used to remove red and dead cells, at pH 5.1 they cannot remove cells killed by antibody and complement whereas at pH 7.2 the gradients give low recoveries of viable cells (Shortman *et al.*, 1972). Furthermore, the low-ionic-strength filtration procedure described by von Boehmer and Shortman (1973) is unable to eliminate dead cells from lymphoid cell cultures and is also unable to deplete red cells from lymphoid cell suspensions.

Thus, the Isopaque/Ficoll procedure represents a good general method for removing red cells and dead cells from cell suspensions. It is simple, and, although slightly more time-consuming than the low-ionic-strength method, it is easier than the albumin-gradient procedure, carefully prepared albumin-density media not being required.

Summary

A procedure is described for simultaneously removing red cells and dead cells from lymphoid cell suspensions, based on the observation that when populations of lymphoid cells are centrifuged on a mixture of Isopaque/Ficoll, dead cells and red cells sediment whereas viable cells float. The technique very efficiently removed red cells from a wide range of lymphoid cell suspensions and eliminated lymphocytes killed by mechanical stress, by antibody and complement and by prolonged tissue culture.

The depletion of red cells was >99% and the recovery of viable lymphocytes usually >90%, the resulting cell suspensions being around 95-100% viable. The immunological activity of B cells, helper T cells and cytotoxic T cells was virtually unimpaired by the separation procedure.

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